Regulation of EGFR Degradation by Ankyrin105

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ABSTRACT

Growth factors can regulate a variety of cellular processes by activating receptors on the cell surface. Many of the receptors belong to the receptor tyrosine kinase family, including the platelet-derived growth factor receptor and the epidermal growth factor receptor and its related family members, such as ErbB2. Constitutively activated receptor tyrosine kinases and their downstream signaling pathways (such as the Ras-mitogen activated protein kinase pathway and the phosphatidylinositol 3 kinase-Akt pathway) are frequently observed in cancer cells. Therefore, understanding the degradation mechanism of receptors is important and may facilitate the development of new prognostic or treatment strategies for cancer. Ankyrin105 is the smaller isoform of ankyrin3 and is localized to late endosomes and lysosomes. Our laboratory has previously shown that ankyrin105 can bind to the phosphatidylinositol 3 kinase regulatory subunit p85, stimulate lysosomal-mediated degradation of the platelet-derived growth factor receptor and differentially affect its signaling pathways in NIH 3T3 cells. To determine whether ankyrin105 can induce degradation of multiple receptor tyrosine kinases in a similar manner, we extended these studies to include the epidermal growth factor receptor and its downstream signaling in this project. Hemagglutinin-tagged ankyrin105 was introduced into COS-1, HEK293T, MCF10A, MDA-MB-231 and AU565 cells, respectively. We demonstrated that overexpression of ankyrin105 did not enhance the epidermal growth factor receptor degradation or downregulation of its signaling pathways in these selected cell lines. AU565 cells, which expressed relatively high levels of both epidermal growth factor receptor and ErbB2, were susceptible to geldanamycin or herceptin facilitated ErbB2 internalization and degradation, which subsequently promoted the epidermal growth factor receptor degradation. However, ankyrin105 did not further improve geldanamycin-induced epidermal growth factor receptor degradation or impact its downstream signaling pathways. These studies suggest that the influence of ankyrin105 may be receptor-specific (platelet-derived growth factor receptor, but not epidermal growth factor receptor) and/or cell type specific (NIH 3T3 cells, but not COS-1, HEK293T, MCF10A, MDA-MB-231 or AU565 cells).

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17-AAG	17-allylamino-17-demethoxygeldanamycin
Ank	ankyrin
AP	adaptor protein
APS	ammonium persulfate
AR	amphiregulin
ATCC	American Type Culture Collection
BAD	Bcl-2/Bcl-X antagonist of cell death
BGH	bovine growth hormone
BH	breakpoint cluster region homology
BSA	bovine serum albumin
BSC	biological safety cabinet
BTC	betacellulin
C2	protein kinase C homology 2
CDC25	cell division cycle 25
CH	collagen homology
CMV	cytomegalovirus
CNK	connector enhancer of KSR
CR	conserved region
CRD	cysteine-rich domain
CUB	C1r/C1s, urchin EGF-like protein and bone morphogenic protein
DH	Dbl homology
DMEM	Dulbecco's Modified Eagle Medium
DMEM/F-12	Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12
DMSO	dimethyl sulfoxide
EDTA	ethylene diamine tetraacetic acid

EEA1	early endosome antigen 1
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
EPHR	ephrin receptor
EPR	epiregulin
ER	estrogen receptor
ERK	extracellular signal-regulated kinase
ESCRT	endosomal sorting complex required for transport
FBS	fetal bovine serum
FCM	flow cytometry
FGF	fibroblast growth factor
FGFR	fibroblast growth factor receptor
GA	geldanamycin
Gads	Grb2-related adapter downstream of Shc
GAP	GTPase activating protein
GDF	GDI-displacement factor
GDI	guanine-nucleotide-dissociation inhibitor
GEF	guanine exchange factor
GFP	green fluorescent protein
GPCR	G protein coupled receptor
G protein	guanine-nucleotide-binding protein
Grap	Grb2-related adapter protein
Grb2	growth factor receptor-bound protein 2
GSK	glycogen synthase kinase
HA	hemagglutinin
HB-EGF	heparin binding EGF-like growth factor
HBS	HEPES buffered saline

HD	helical domain
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HGF	hepatocyte growth factor
HGFR	hepatocyte growth factor receptor
HIP	heat shock cognate protein 70-interacting protein
HOP	Hsp40/Hsp70 organizing protein
HRG	heregulin
Hsp	heat shock protein
HSV	herpes simplex virus
HVR	hypervariable region
IGF	insulin-like growth factor
IGFR	insulin-like growth factor receptor
IR	insulin receptor
JAK	Janus kinase
KSR	kinase suppressor of Ras
LGB	lower gel buffer
MAGI	membrane-associated guanylate kinase with inverted organization
MAPK	mitogen activated protein kinase
MEK	MAPK kinase/ ERK kinase
MEM	Minimum Essential Media
MOI	multiplicity of infection
mTORC2	mammalian target of rapamycin complex 2
MVB	multivesicular body
NGF	nerve growth factor
NGFR	nerve growth factor receptor
NHERF	Na^+/H^+ exchanger regulatory factor
NRG	neuregulin

PAGE	polyacrylamide gel electrophoresis
PBD	$PI4,5P_2$ binding domain
PBS	phosphate buffered saline
PBST	phosphate buffered saline Tween-20
PDGF	platelet-derived growth factor
PDGFR	platelet-derived growth factor receptor
PDK	phosphoinositide-dependent kinase
PDZB	PSD-95/Dlg/ZO-1 binding
PE	phycoerythrin
PFU	plaque forming units
PH	pleckstrin homology
PI3K	phosphatidylinositol 3 kinase
PI3P	phosphatidylinositol 3-phosphate
$PI3,4,5P_3$	phosphatidylinositol 3,4,5 trisphosphate
$PI4,5P_2$	phosphatidylinositol 4,5 bisphosphate
PKB	protein kinase B
PLC γ	phospholipase C γ
PR	progesterone receptor
PTB	phosphotyrosine binding
PTEN	phosphatase and tensin homolog deleted on chromosome 10
RBD	Ras-binding domain
REM	Ras exchange motif
RPMI 1640	Roswell Park Memorial Institute Medium 1640
RTK	receptor tyrosine kinase
SDS	sodium dodecyl sulfate
SH2	Src homology-2
SH3	Src homology-3

Shc	Src homologous and collagen
SNARE	soluble N-ethylmaleimide sensitive factor attachment receptor
Sos	son of sevenless
STAT	signal transducer and activator of transcription
SV40	Simian Vacuolating Virus 40
TEMED	N,N,N’,N’-tetramethylethylenediamine
TF	transcription factor
TGF	transforming growth factor
TK	thymidine kinase
TKI	tyrosine kinase inhibitor
TLR	toll-like receptor
TR	tomoregulin
Tris	tris (hydroxymethyl) aminomethane
UGB	upper gel buffer
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor
WB	Western blot

1.0 INTRODUCTION

1.1 Overview of Signal Transduction

Signal transduction is a process that whereby extracellular signaling molecules activate specific receptors, leading to the transmission of signals into the cell and the regulation of cellular processes. It can regulate gene expression, enzyme activity, metabolism and many other processes. The signaling molecules participate in most cellular functions and are thus responsible for physiological changes in the organism. Defects in signal transduction may result in cancers and other diseases.

Most extracellular signaling molecules are not able to transit across the cell membrane directly because of their large size and hydrophilic polarity (Berg *et al*., 2002). So membrane-associated receptors act as intermediates for signal transduction. When an extracellular signaling molecule targets to its receptor on the cell surface, the receptor recruits a variety of intracellular molecules to complete this process. Many of the receptors are transmembrane proteins, such as receptor tyrosine kinases (RTKs), G protein coupled receptors (GPCRs), integrins and toll-like receptors (TLRs) (Berg *et al*., 2002). Among these receptors, RTKs are related to this project.

The extracellular domain of RTKs recognizes and binds to the extracellular growth factors and then the intracellular domain is activated. Protein phosphorylation cascades are carried out to transfer the signal inside cells and regulate cellular processes. They are involved in several signaling pathways, including the Ras-mitogen activated protein kinase (MAPK) pathway, the phosphatidylinositol 3 kinase (PI3K)-Akt pathway, the phospholipase C γ (PLC γ) pathway, the Janus kinase (JAK)-signal transducers and activators of transcription (STATs) pathway (Biarc *et al*., 2011; Dengjel *et al*., 2009; Madhani, 2001).

Since continuously activated RTKs and signaling pathways are correlated with abnormalities in cells, the signal transduction needs to be terminated soon after activation. Therefore, the downregulation mechanism of RTKs is important. Activated RTKs are rapidly

endocytosed mainly via a clathrin-dependent or a clathrin-independent manner (Mousavi *et al*., 2004; Mukherjee *et al*., 1997). Many of the receptors are dephosphorylated and recycled back to the cell surface. Only a small fraction of receptors may divert to lysosomes for degradation (Sorkin and Von Zastrow, 2002).

1.2 Receptor Tyrosine Kinases

Growth factors can control a variety of cellular processes by activating corresponding receptors on the cell surface. They are very important in regulating cell growth, cell cycle control, metabolism, proliferation, differentiation, maturation, survival and migration (Bache *et al*., 2004; Lemmon and Schlessinger, 2010). Most of them are proteins or steroid hormones. Growth factor are divided into many classes, including epidermal growth factor (EGF), platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), hepatocyte growth factor (HGF), nerve growth factor (NGF), vascular endothelial growth factor (VEGF), insulin, insulin-like growth factor (IGF) and ephrin (Aaronson, 1991).

Many of the growth factor receptors belong to the RTK family. Fifty eight RTKs have been identified in the human genome (Robinson *et al*., 2000). RTKs are composed of an N-terminal extracellular ligand-binding domain, a single transmembrane domain and a highly conserved C-terminal cytoplasmic tyrosine kinase domain (Biarc *et al*., 2011; Cowan-Jacob, 2006; Li and Hristova, 2010; Takeuchi and Ito, 2011). According to the binding ligand and specific structural differences, RTKs can be divided into 20 subfamilies. These include epidermal growth factor receptor (EGFR), platelet-derived growth factor receptor (PDGFR), fibroblast growth factor receptor (FGFR), hepatocyte growth factor receptor (HGFR), nerve growth factor receptor (NGFR), vascular endothelial growth factor receptor (VEGFR), insulin receptor (IR), insulin-like growth factor receptor (IGFR) and ephrin receptor (EPHR). The structures of these members differ by their extracellular and tyrosine kinase domains (Figure 1.1) (Aaronson, 2005; Blume-Jensen and Hunter, 2001; Lemmon and Schlessinger, 2010; Robertson *et al*., 2000).

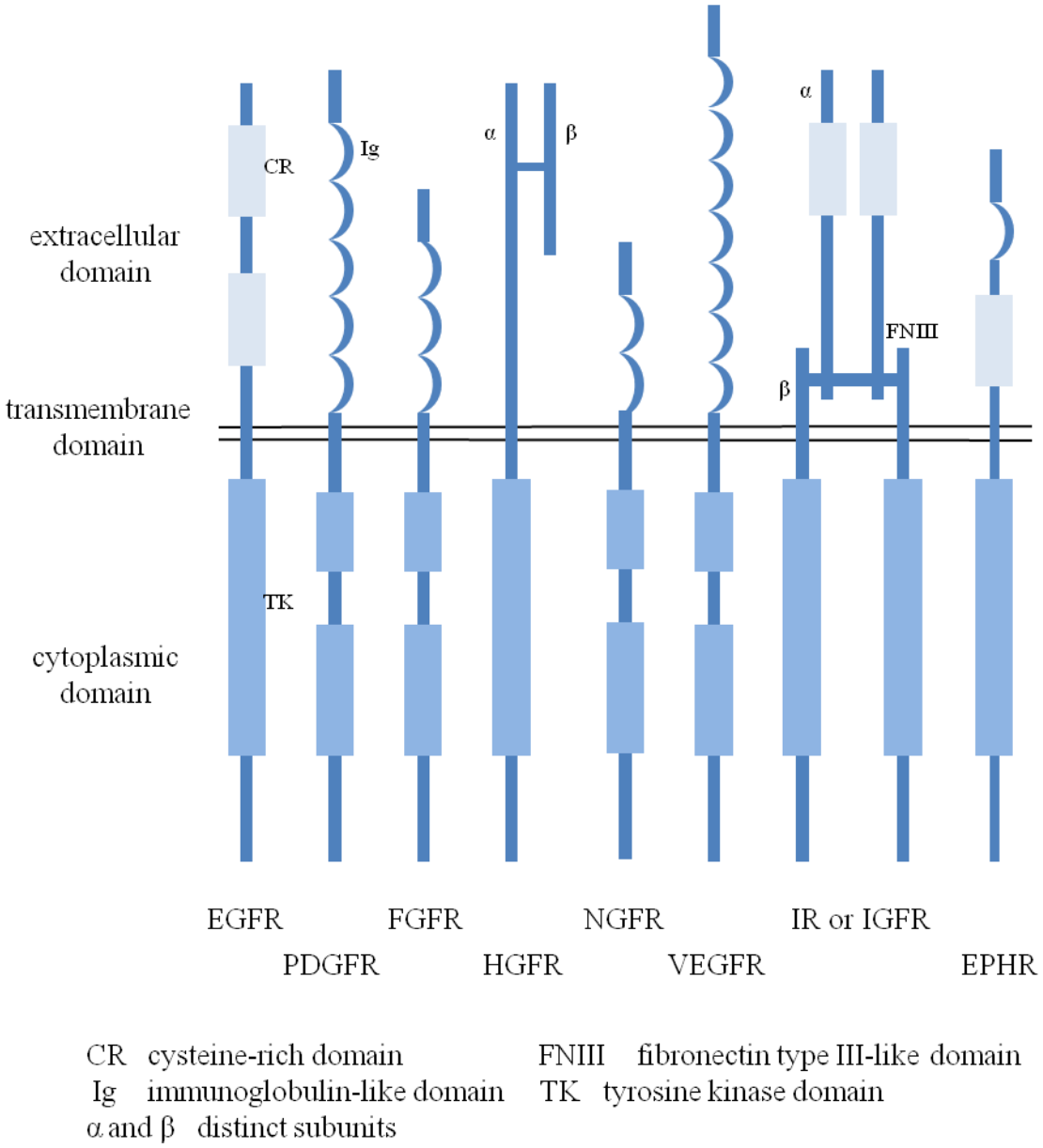

Figure 1.1 The structures of major members of different receptor tyrosine kinase families. RTKs are composed of an N-terminal extracellular ligand-binding domain, a single transmembrane domain and a C-terminal cytoplasmic tyrosine kinase domain. The structures of these members are different within the extracellular and tyrosine kinase domains. The extracellular domain of EGFR, IR, IGFR and EPHR has one or two cysteine-rich domains and the extracellular domain of PDGFR, FGFR, NGFR, VEGFR and EPHR has one or more immunoglobulin-like domains. The tyrosine kinase domain of PDGFR, FGFR, NGFR and VEGFR is divided by a non-catalytic kinase insert domain. The figure is modified from Blume-Jensen and Hunter, 2001 and Lemmon and Schlessinger, 2010.

Growth factors and RTKs are essential for signal transduction inside cells. Extracellular signals (growth factors) can trigger a series of intracellular signaling pathways via the transmembrane receptors (RTKs). Multiple RTKs share a similar mechanism to be activated and then activate downstream signaling pathways. An "open-closed" switch mechanism has been demonstrated in EGFR and FGFR (Burgess *et al*., 2003; Olsen *et al*., 2004). The extracellular domain is inactive without growth factors, which suggests a "closed" state. When ligands (growth factors) bind to the extracellular domain of RTKs, the dimerization region within the extracellular domain is exposed and the receptor dimerizes with the neighboring receptor, which is referred as the "open" state (Bae and Schlessinger, 2010; Burgess *et al*., 2003; Li and Hristova, 2006; Olsen *et al*., 2004). In summary, ligand docking regulates the RTK equilibrium between "open" and "closed" state. Ligand binding causes a conformational change of the extracellular domain and dimer formation. However, there are some exceptions. ErbB2, which is from the EGFR subfamily, has a constitutively exposed dimerization arm and thus it is able to form dimers with its subfamily members in the absence of growth factors (Burgess *et al*., 2003). IR or IGFR is a constitutive dimer, linked by disulfide bonds, and ligand binding activates the tyrosine kinase (Ottensmeyer *et al*., 2000). In some studies, the transmembrane domain also contributes to the dimerization in EGFR and FGFR (Li *et al*., 2005; Mendrola *et al*., 2002). In an unstimulated RTK, an activation loop blocks the tyrosine kinase domain in the cytoplasm from its potential substrates. Upon ligand docking, the tyrosine residues in the activation loop are autophosphorylated by its own tyrosine kinase domain due to the adjacent dimeric receptor (Hubbard and Till, 2000). The phosphorylation of the activation loop releases itself from the tyrosine kinase domain and stabilizes its own conformation. This fully activated receptor then phosphorylates additional tyrosine residues within the intracellular domain (Bae and Schlessinger, 2010; Blume-Jensen and Hunter, 2001; Robertson *et al*., 2000). This provides several binding sites for many intracellular signaling molecules and some are phosphorylated by the receptor. The binding of signaling molecules to the receptor phosphotyrosine sites is usually via Src homology-2 (SH2) domain or phosphotyrosine-binding (PTB) domain in the

signaling proteins (Zhou, 2005). Downstream signaling pathways are then activated, such as the Ras-MAPK pathway and the PI3K-Akt pathway as described in section 1.3 (Biarc *et al*., 2011; Dengjel *et al*., 2009).

Mutation and deregulation of RTKs are usually correlated to a variety of human syndromes and diseases, including cancers (Aaronson, 1991; Lemmon and Schlessinger, 2010; Robertson *et al*., 2000). For example, mutations in IR may result in type A insulin resistance (Ottensmeyer *et al*., 2000) and mutations in FGFR may cause Dwarfism syndromes (Webster and Donoghue, 1997). Overexpression and defective downregulation of EGFR have been linked to several cancers, such as breast cancer, colorectal carcinoma, lung cancer and brain tumors (Klapper *et al*., 2000; Yarden and Sliwkowski, 2001). Mutations in the extracellular domain and the tyrosine kinase domain, directly affect RTK signaling. Whereas, it has been demonstrated that mutations in the transmembrane domain also plays a critical role in RTK function. They may impact ligand binding, receptor dimerization, the structure of the tyrosine kinase domain and downregulation of activated RTK (Li and Hristova, 2010). Therefore, RTKs are used as useful targets for drug research and development for the treatment of cancers and other RTK related diseases (Takeuchi and Ito, 2011; Wieduwilt and Moasser, 2008).

1.2.1 Epidermal Growth Factor Receptor

The EGFR subfamily is the most well studied among RTK families and it contains 4 members: EGFR (ErbB1 or HER1), ErbB2 (HER2 or Neu), ErbB3 (HER3) and ErbB4 (HER4) (Burgess, 2008). They can regulate embryonic development, cell lineage determination, tissue repair and cancer. The EGFR subfamily was the first discovered RTK in cancer and the members are the most studied receptors for understanding carcinogenic mechanism (Burgess, 2008; Eccles, 2011; Pinkas-Kramarski *et al*., 1997).

The EGFR is activated by the dimerization of its family members via ligand binding. They can form both homodimers and heterodimers with different family members. At least 13 ligands have been discovered (Yarden, 2001). EGFR and ErbB4 have a number of ligands, while no

ligand is found to bind to ErbB2 (Citri *et al*., 2003). EGF, transforming growth factor-α (TGF-α), heparin binding EGF-like growth factor (HB-EGF), amphiregulin (AR), betacellulin (BTC), epigen and epiregulin (EPR) are EGFR ligands. ErbB3 only has 3 ligands: neuregulin (NRG) 1, NRG 2 and heregulin (HRG). ErbB4 ligands include HB-EGF, BTC, EPR, tomoregulin (TR), NRG 1, NRG 2, NRG 3, NRG 4 and HRG. All of these growth factors have a common EGF motif, which consists of 6 cysteine residues including 3 intramolecular disulfide bonds. This motif plays a key role in ligand binding. These ligands are present as type I transmembrane proteins, and mature growth factors are generated by enzyme cleavage at the cell surface (Berasain *et al*., 2007; Harris *et al*., 2003; Ogiso *et al*., 2002).

Like other RTKs, EGFR members share a similar basic structure: an extracellular ligand-binding domain (about 620 residues), a hydrophobic transmembrane domain (about 23 residues) and a highly conserved cytoplasmic tyrosine kinase domain (about 260 residues). There is a C-terminal regulatory region (about 232 residues) containing multiple tyrosine residues preceded by the kinase domain (Figure 1.2) (Burgess *et al*., 2003). The extracellular region is highly glycosylated (Zhen *et al*., 2003) and has 4 distinct subdomains: 2 homologous large domains (domain I or L1 and III or L2) that contain leucine-rich repeats and 2 cysteine-rich domains (domain II or S1 and IV or S2) that may have several disulfide-bonded modules (Figure 1.3 A). A crystal structure study shows the EGF-EGFR and EGFR-EGFR interactions, which reveals the dimerization mechanism of EGFR (Figure 1.4) (Ogiso *et al.*, 2002). One EGF molecule binds to one EGFR molecule and then two 1:1 EGF-EGFR complexes form a dimer. The ligand binding region is between domains I and III. Domain II is characterized as the dimerization arm, which is a unique feature for EGFR subfamily. The conformational change after ligand docking breaks the link between domains II and IV, exposes the dimerization arm, and then induces dimerization (Figure 1.3 B) (Burgess *et al*., 2003; Ogiso *et al*., 2002).

The intracellular tyrosine kinase domain is functionally inactive when the receptor is still a monomer. Since homodimers or heterodimers are formed upon ligand stimulation, the adjacent

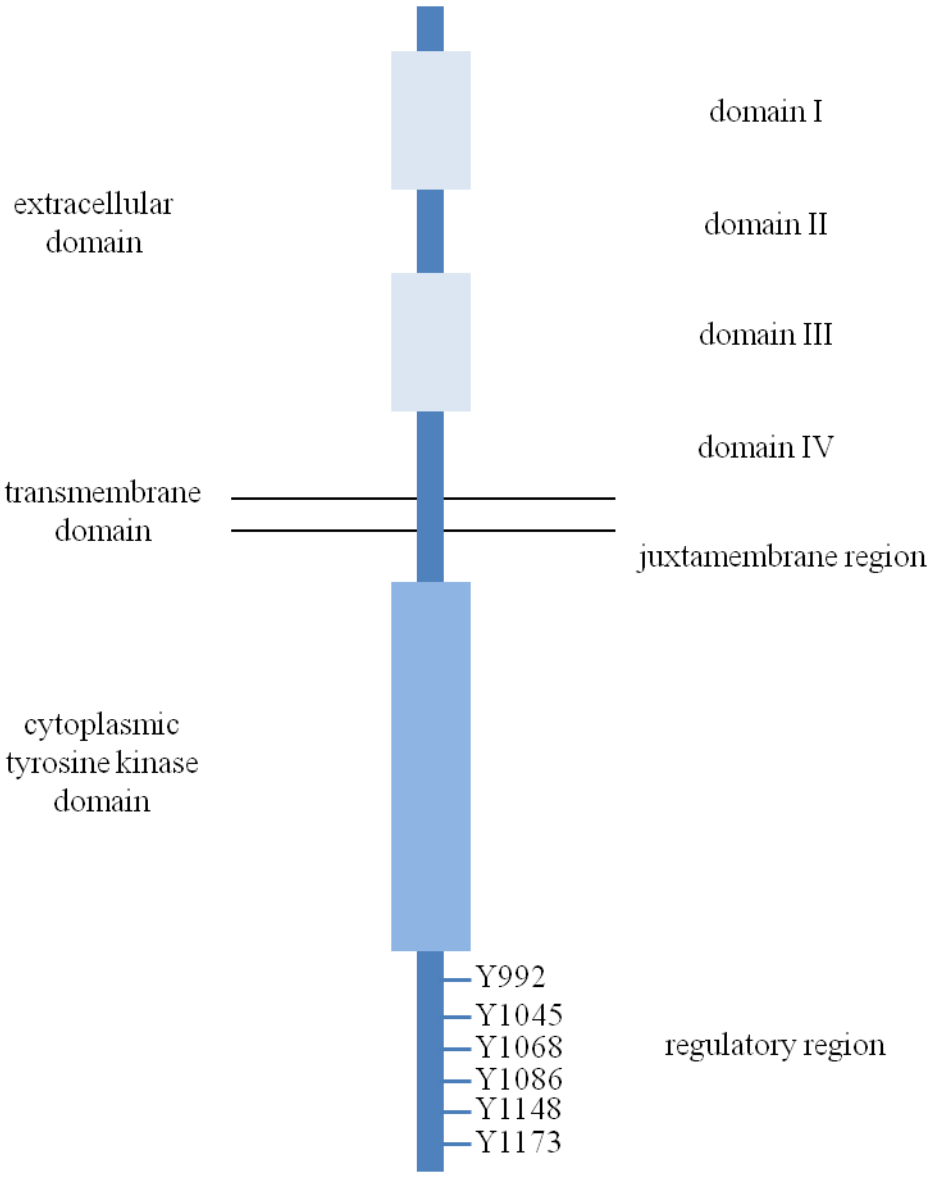

Figure 1.2 The domain structure of EGFR subfamily. There are 3 major domains: an extracellular domain, a transmembrane domain and a cytoplasmic tyrosine kinase domain. The extracellular domain has 4 distinct subdomains: domain I, II, III and IV. The intracellular tyrosine kinase domain is between the juxtamembrane region and C-terminal regulatory region containing 6 tyrosine residues that can be phosphorylated. The figure is modified from Bazley and Gullick, 2005 and Burgess *et al*., 2003.

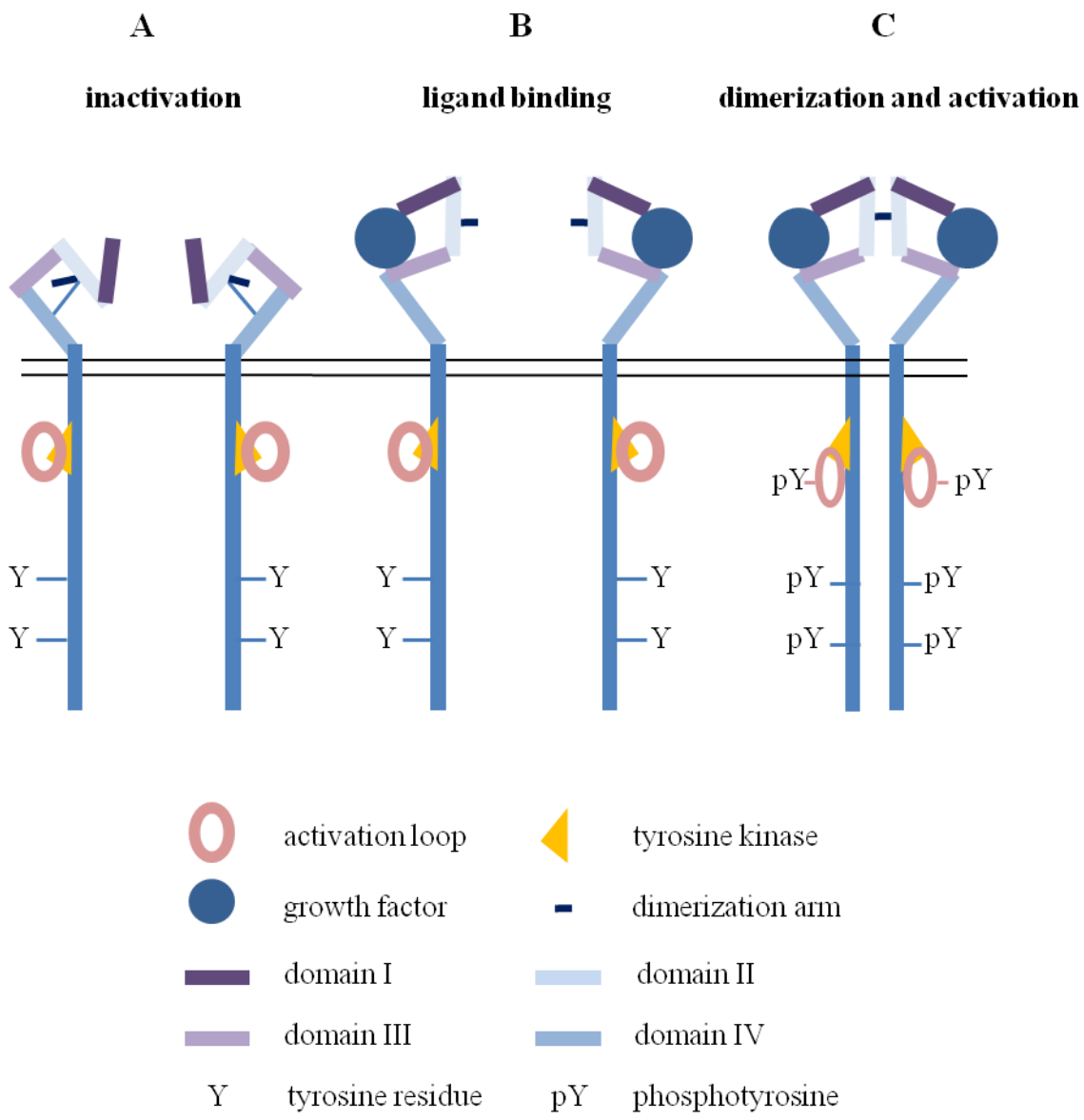

Figure 1.3 Growth factors induce activation of EGFR. A, Domains II and IV are linked to each other and the tyrosine kinase domain is inactive before ligand binding. **B,** The conformational change after ligand docking breaks domains II and IV link, exposes the dimerization arm in domain II and then induces dimerization. **C,** The dimerization promotes the autophosphorylation of activation loop. The phosphorylation of the activation loop releases itself from the tyrosine kinase domain and stabilizes its own conformation, and facilitates phosphorylation of tyrosine residues within the intracellular domain. The figure is modified from Mpofu, 2010 and Burgess *et al*., 2003.

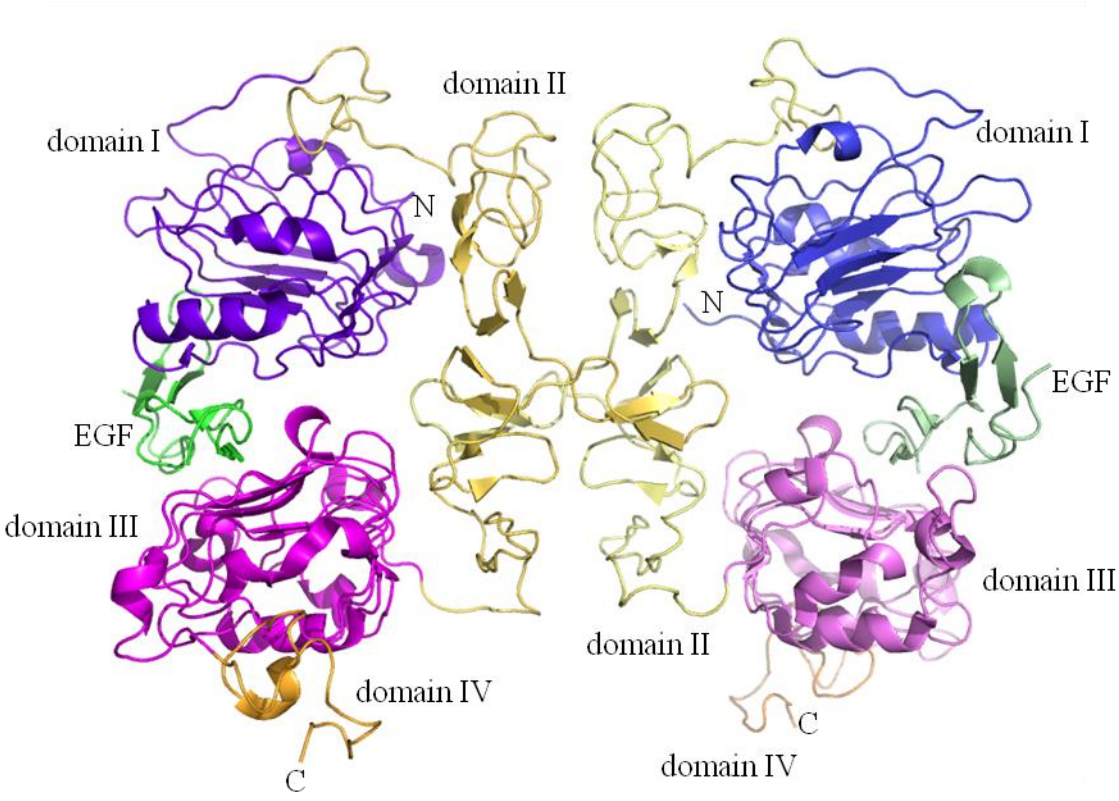

Figure 1.4 The crystal structure of EGF-EGFR complex. The original structure data of EGF-EGFR complex was obtained from Protein Data Bank accession number 1IVO (Ogiso *et al.*, 2002). The ribbon diagram was developed using PyMOL software (version 1.4.1). EGF and the extracellular subdomains I, II, III and IV of EGFR were labeled in different colors. EGF-EGFR and EGFR-EGFR interactions are shown. The EGF binding region is between domains I and III of the receptor. Domain II plays a role in EGFR dimerization.

2 tyrosine kinase domains promote the autophosphorylation of the activation loop and thereby activate the intrinsic catalytic activity of the tyrosine kinase domain (Bazley and Gullick, 2005; Fuller *et al*., 2008; Hubbard and Till, 2000). Some tyrosine residues within the regulatory region are then phosphorylated (Figure 1.2 and Figure 1.3 C). Every EGFR subfamily member has its own phosphotyrosine profile, which dictates the different downstream signaling molecules recruited (Pedersen *et al*., 2009). In addition, there is a juxtamembrane region (about 40 residues) between the transmembrane and the intracellular domains (Figure 1.2) (Burgess *et al*., 2003). It can autoinhibit the tyrosine kinase activity and negatively regulate tyrosine phosphorylation (Hubbard, 2004).

EGFR, the first identified and widely expressed member in this subfamily, is a 180 kDa glycoprotein. It was shown that all other EGFR subfamily members evolved from the EGFR (Sithanandam and Anderson, 2008). Normally, EGFRs are rapidly internalized and endocytosed once activated (Pedersen *et al*., 2009). Overexpression of EGFR impairs this downregulation process (Worthylake *et al*., 1999). Therefore, mutations and upregulation of EGFR may contribute to tumorigenesis.

ErbB2 is a unique member in several aspects. First of all, no known ligand has been identified that can bind to ErbB2. Secondly, the extended structure of extracellular domain constitutively exposes the dimerization arm, so ErbB2 can form dimers without ligand docking. ErbB2 may be constitutively activated or be activated by dimerization. Thirdly, ErbB2 is a potential heterodimerization partner and ErbB2 overexpression may promote heterodimerization with other EGFR subfamily members because no ligand is required. Some studies suggest that there is a preference for forming heterodimers rather than homodimers for ErbB2, and the ErbB2-ErbB3 heterodimer is the most widely represented and most active (Burgess *et al*., 2003; Citri *et al*., 2003; Fuller *et al*., 2008; Yarden and Sliwkowski, 2001). Furthermore, ErbB2 is internalization resistant, and thus the heterodimers containing ErbB2 are very stable and their endocytosis is inhibited, which indicates that the downstream signaling is prolonged (Pedersen *et al*., 2009). ErbB2 can also enhance the binding affinity of the ligand to

its dimeric receptor (Fuller *et al*., 2008). Therefore, overexpression of ErbB2 is detrimental for cells and normally linked to cancers (Yarden and Sliwkowski, 2001). It has been shown that about 20% to 30% of breast cancer results from ErbB2 overexpression (Pedersen *et al*., 2008).

ErbB3 can bind to its ligands but does not have tyrosine kinase activity due to the absence of some critical residues in its intracellular domain. However, the tyrosine residues in the regulatory region can still be phosphorylated and downstream signaling pathways can be triggered via the dimeric partner receptor in the ErbB3 heterodimer or by other kinases (Citri *et al*., 2003; Fuller *et al*., 2008).

ErbB4 is the most related member to EGFR and is also fully functional (Wieduwilt and Moasser, 2008). Only ErbB4 has different isoforms and these isoforms are tissue specific (Veikkolainen *et al*., 2011) and critical in cardiovascular and neural development (Junttila *et al*., 2000).

1.2.2 Platelet-Derived Growth Factor Receptor

PDGFR subfamily has 2 members: PDGFR α (170 kDa) and PDGFR β (190 kDa) (Claesson-Welsh, 1994). They have the RTK structural backbone and can homodimerize or heterodimerize with each other. The extracellular domain consists of 5 immunoglobulin-like domains and the first 3 domains are involved in ligand binding. However, domain 2 seems to play the most important role in binding. Domain 4 may help stabilize the PDGFR dimer (Heldin and Westermark, 1999). There is about 31% identity in the PDGFR α and PDGFR β ligand-binding domain. The tyrosine kinase domain is divided by a kinase insert domain in cytoplasmic region. This insert domain does not have catalytic activity. Multiple tyrosine residues that can be phosphorylated reside in juxtamembrane region, kinase insert domain and regulatory region (Figure 1.5) (Grimminger and Schermuly, 2010; Jones and Cross, 2004; Yu *et al*., 2003).

The PDGF family, the PDGFR ligand, has 4 different peptide chains (PDGF-A, PDGF-B, PDGF-C and PDGF-D) encoded by 4 distinct genes (Tallquist and Kazlauskas, 2004). They all

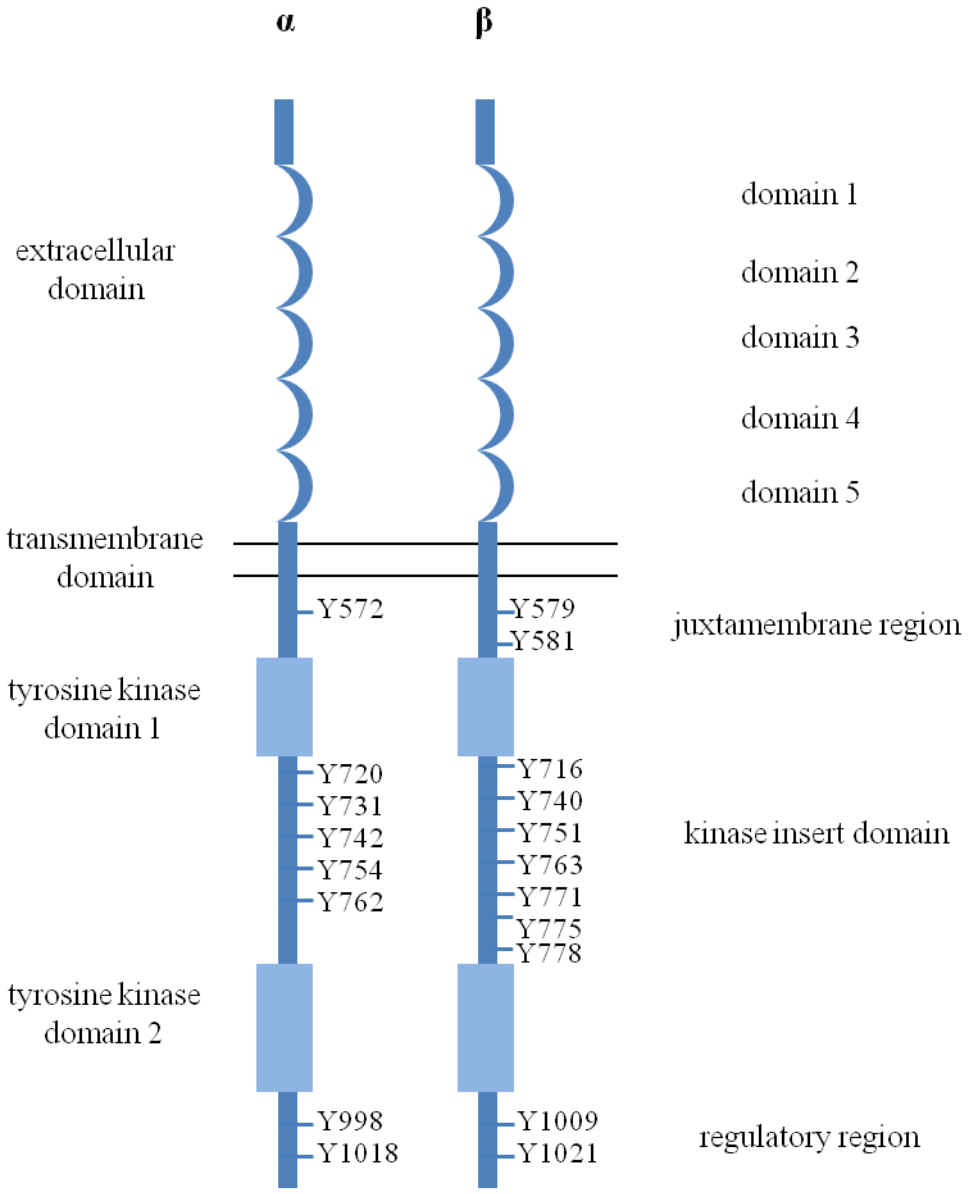

Figure 1.5 The domain structures of PDGFR α and PDGFR β. The extracellular domain consists of 5 immunoglobulin-like domains. The cytoplasmic region has a split kinase domain interrupted by a kinase insert domain. There are multiple tyrosine residues that can be phosphorylated in the juxtamembrane region, kinase insert domain and regulatory region. The figure is modified from Chamberlain, 2007 and Jones and Cross, 2004.

function as dimers, and a dimer can bind to and activate 2 PDGFRs simultaneously (Heldin and Westermark, 1999). Compared to PDGF-A and PDGF-B, PDGF-C and PDGF-D are recently discovered and have a unique N-terminal C1r/C1s, urchin EGF-like protein and bone morphogenic protein 1 (CUB) domain other than the common growth factor domain. Five homodimers or heterodimers are formed within these isoforms by a disulfide bond. PDGF-A and PDGF-B can both homodimerize (PDGF-AA and PDGF-BB) and heterodimerize (PDGF-AB) with each other, but PDGF-C and PDGF-D can only form homodimers (PDGF-CC and PDGF-DD) (Reigstad *et al*., 2005). PDGF-AA, PDGF-AB, PDGF-BB and PDGF-CC can stimulate PDGFR α, whereas PDGF-BB and PDGF-DD can activate PDGFR β. In addition, PDGF-AB, PDGF-BB and PDGF-CC can bind to and then activate PDGFR αβ heterodimers (Board and Jayson, 2005; Claesson-Welsh, 1996; Fredriksson *et al*., 2004).

When PDGF binds to the extracellular domain of PDGFRs, receptors dimerize and cause conformational change. The following steps are the same as for other RTKs: tyrosine kinase activation and receptor autophosphorylation. Phosphotyrosines are the binding sites for many intracellular signaling proteins containing SH2 and PTB domains. Thus, downstream signaling pathways are activated (Claesson-Welsh, 1994; Grimminger and Schermuly, 2010; Jones and Cross, 2004).

PDGFR can control embryonic development, cell migration, survival, proliferation and angiogenesis. Abnormalities of PDGFR are involved in some diseases and cancers (Jones and Cross, 2004; Yu *et al*., 2003). For example, overexpression of PDGFRs may facilitate prostate cancer cell metastases to bone and overexpression of PDGFR α is associated with high mortality rate in ovarian cancer (Board and Jayson, 2005). Increased PDGF signaling via PDGFR β is observed in some vascular diseases, such as atherosclerosis and pulmonary hypertension (Dai, 2010).

1.3 Signaling Pathways

When growth factors bind to RTKs, the receptors are phosphorylated and recruit many

intracellular signaling molecules. Several signaling pathways are then activated. The Ras-MAPK pathway and the PI3K-Akt pathway are the most important ones.

1.3.1 The Ras-Mitogen Activated Protein Kinase Signaling Pathway

Src homologous and collagen (Shc) adaptor family has 4 members: Shc (ShcA), ShcB (Sli) ShcC (Rai or N-Shc) and ShcD (RaLP) (Finetti *et al*., 2009). ShcA has 3 isoforms of 44, 52, and 66 kDa. p52 ShcA has been identified to couple RTKs to Ras-MAPK pathway to transfer activated tyrosine phosphorylation signaling. Unlike other isoforms, it is widely expressed in the cytoplasm and translocated to the plasma membrane after growth factor stimulation (Alam *et al*., 2009). p52 ShcA has an N-terminal PTB domain that can bind to the phosphotyrosine of RTK, a central proline-rich collagen homology (CH) 1 domain that includes 3 conserved tyrosine residues (Y239, Y240 and Y317) and a C-terminal SH2 domain that might also be responsible for binding to activated RTKs (Figure 1.6) (Cattaneo and Pelicci, 1998; Ravichandran, 2001; Zhang *et al*., 2003). These tyrosine residues in the CH1 domain can be phosphorylated by activated RTKs and become binding sites for other SH2 containing proteins. In addition, proteins including Src homology-3 (SH3) domain can target to proline-rich sequence in CH1 domain (Pellegrini *et al*., 2005).

Growth factor receptor-bound protein 2 (Grb2) adaptor family has 3 members: Grb2, Grb2-related adapter protein (Grap) and Grb2-related adapter downstream of Shc (Gads) (Jang *et al*., 2009). Grb2 is a ubiquitously expressed cytoplasmic protein of 25 kDa and its central SH2 domain is flanked by 2 SH3 domains (Figure 1.6). It functions as a target for sequences containing either phosphotyrosine or abundant proline residues (Giubellino *et al*., 2008). Its SH2 domain can not only directly bind to activated RTK, but also interact with intracellular proteins containing specific phosphotyrosine, such as Y317-phosphorylated p52 ShcA (Cattaneo and Pelicci, 1998; Zhang *et al*., 2003). Its SH3 domains can bind to proline-rich proteins. For example, son of sevenless (Sos) 1 is constitutively linked to Grb2 (Giubellino *et al*., 2008).

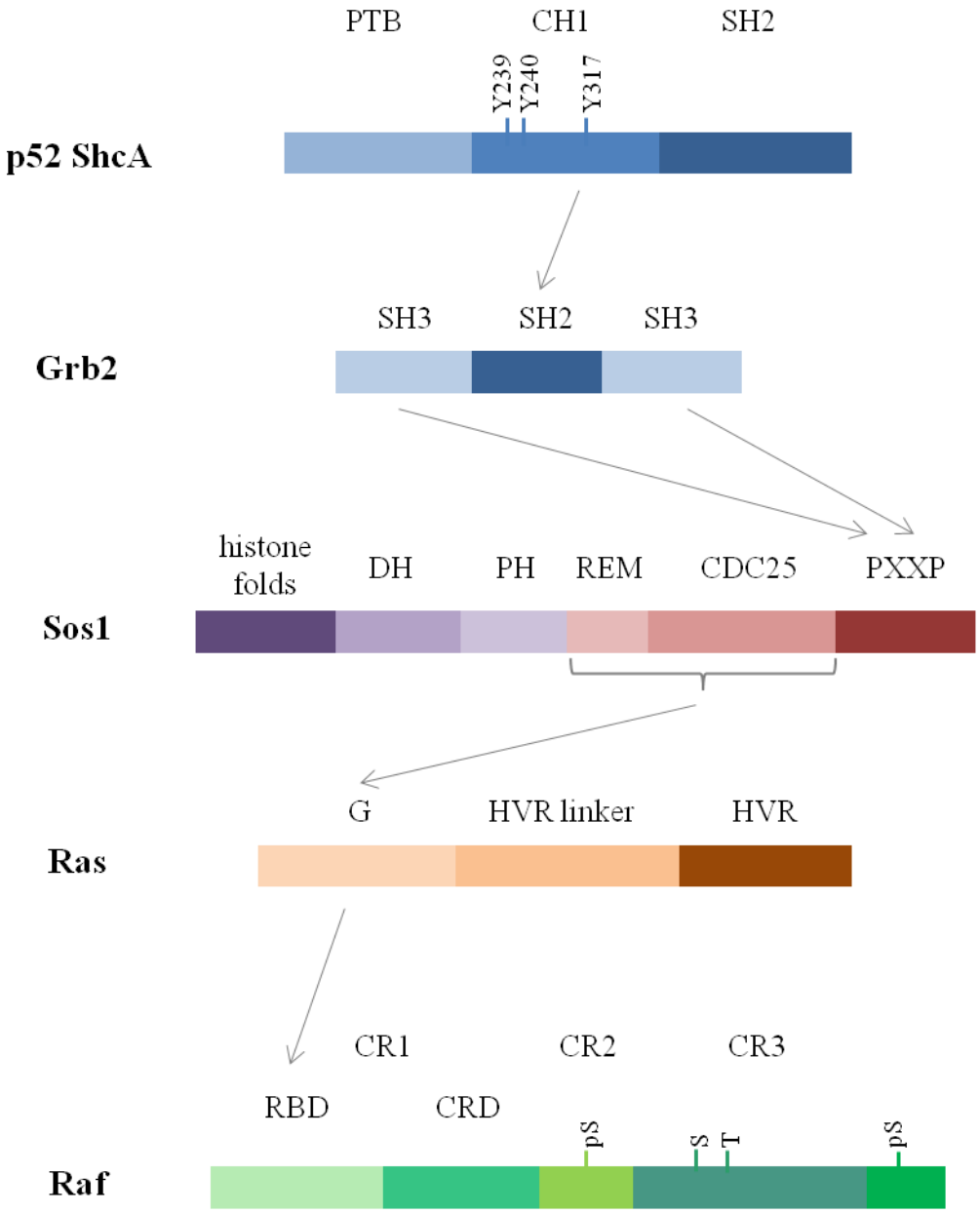

Figure 1.6 The domain structures of several intracellular signaling proteins involved in the Ras-MAPK pathway. These proteins are p52 ShcA, Grb2, Sos1, Ras and Raf. The arrows show the interaction between domains of these proteins.

Sos has 2 identified human homologues and Sos1 (150 kDa) is a cytoplasmic Ras-specific guanine exchange factor (GEF) that activates Ras by converting GDP-bound Ras to GTP-bound Ras (Pierre *et al*., 2011; Watanabe *et al*., 2000). The N-terminal region of Sos has 2 tandem histone folds that can interact with the plasma membrane and control Sos1 function. The Dbl homology (DH) and pleckstrin homology (PH) domains are reported to negatively regulate Ras activation by blocking allosteric Ras binding site. Upon growth factor stimulation, Sos1 is recruited to the plasma membrane by forming Shc-Grb2-Sos complex and the autoinhibition is released to activate Sos. The Ras exchange motif (REM) and cell division cycle 25 (CDC25) domains can interact with GDP-bound Ras and provide the catalytic activity towards Ras to facilitate GDP for GTP exchange. The C-terminal region containing a specific P-X-X-P proline-rich sequence is responsible for binding to proteins containing SH3 domain, such as Grb2 (Figure 1.6) (Freedman *et al*., 2006; Pierre *et al*., 2011; Rojas *et al*., 2011; Sondermann *et al*., 2004).

Ras, a 20-25 kDa small guanine-nucleotide-binding protein (G protein), is a member from the small GTPase superfamily and consists of H-Ras, K-RasA, K-RasB and N-Ras isoforms (Fehrenbacher *et al*., 2009; Rojas *et al*., 2012). It is localized to the plasma membrane by a lipid anchor after post-translational modification of the C-A-A-X sequence in the C-terminal hypervariable region (HVR) (Figure 1.6). The HVR linker domain may also contribute to the membrane localization. The N-terminal G domain is the guanine-nucleotide-binding site, which is the functional domain for GDP/GTP exchange (Abankwa *et al*., 2007; Eisenberg and Henis, 2008; Fehrenbacher and Philips, 2009). Ras is a molecular switch cycled between 2 conformations: active GTP-bound state and inactive GDP-bound state (Bivona and Philips, 2003). GEFs, such as Sos1, bind to the GDP-bound state. Sos1 catalyzes bound GDP dissociation from the guanine nucleotide binding pocket of Ras and then GTP preferentially binds to the pocket because GTP is 10-fold more abundant than GDP in cytosol (Ahearn *et al*., 2012). Thus, Ras is activated by GEFs due to the conformational change in the GTP-bound state. Its effector domain (G domain) can only trigger downstream signaling in the active

GTP-bound state (Avruch *et al*., 2001; Day *et al*., 1998; Fehrenbacher *et al*., 2009; Mor and Philips, 2006). GTPase activating proteins (GAPs) can deactivate Ras by activating the weak intrinsic GTPase activity of Ras to hydrolyze GTP to GDP, which can negatively regulate the Ras mediated signaling pathway (Bos *et al*., 2007; Vigil *et al*., 2010).

Raf is a cytoplasmic serine/threonine protein kinase composed of 3 members: A-Raf, B-Raf, and C-Raf (Raf-1). B-Raf has high basal kinase activity compared to the other 2 members (Maurer *et al*., 2011). Raf has 3 conserved regions (CRs) and multiple phosphorylation sites (Figure 1.6). The N-terminal CR1 is a regulatory domain that contains a Ras-binding domain (RBD) and a cysteine-rich domain (CRD) that interacts with the CR3 kinase domain to inhibit kinase activity. 14-3-3 protein dimers bind to a specific phosphoserine site in the CR2 serine/threonine-rich domain to stabilize the inactive state and negatively control Raf activation (Leicht *et al*., 2007; Sridhar *et al*., 2005; Wellbrock *et al*., 2004). The effector domain of GTP-bound Ras binds to the RBD of Raf and relocalizes Raf to the plasma membrane. In addition, the specific serine residue in CR2 is dephosphorylated and 14-3-3 proteins are dissociated upon Ras binding. Raf activation is through phosphorylation of multiple residues. There is another 14-3-3 binding site in the C-terminus of Raf, which is involved in positive regulation of the kinase activity (Beeram *et al*., 2005; Claperon and Therrien, 2007; Udell *et al*., 2011). In addition, it is suggested that Raf homodimerization or heterodimerization is also required for Raf activation (Matallanas *et al*., 2011; Roskoski, 2010). Once the GTP is hydrolyzed to GDP in Ras, Raf is released and becomes inactive (Leicht *et al*., 2007).

Mitogen activated protein kinase (MAPK) kinase/extracellular signal-regulated kinase (ERK) kinase (MEK) is a dual specificity kinase in cytoplasm that can phosphorylate threonine and tyrosine and is highly specific to MAPK (Shaul and Seger, 2007; Trujillo, 2011). There are 2 isoforms: 45 kDa MEK1 and 46 kDa MEK2, which are about 80% identical and function equally in activation of MAPK (Shaul and Seger, 2007). Activated Raf can phosphorylate 2 serine residues (Ser218 and Ser222 on MEK1, Ser222 and Ser226 on MEK2) in the

S-X-A-X-S/T motif in the activation loop of MEK and then activate MEK (Chapman and Miner, 2011; Keshet and Seger, 2010; Shaul and Seger, 2007; Yoon and Seger, 2006).

MAPK (ERK) is a cytoplasmic serine/threonine protein kinase with 2 isoforms: 42 kDa MAPK1 (ERK2) and 44 kDa MAPK3 (ERK1), that share about 70% similarity (Shaul and Seger, 2007). It can be phosphorylated on a threonine residue and a tyrosine residue (Thr183 and Tyr185 in MAPK1, Thr202 and Tyr204 in MAPK3) located in the T-G-Y motif in the activation loop of MAPK and thus activated by MEK (Chang *et al*., 2003b; Keshet and Seger, 2010; McCubrey *et al*., 2007; Rubinfeld and Seger, 2005). The substrates of MAPK include transcription factors (TFs), kinases, phosphatases, receptors and scaffold proteins. They have a consensus P-S/T-P or S/T-P sequence that can be phosphorylated because the kinase activity of MAPK is proline-directed. Activated MAPK can translocate into the nucleus and phosphorylate TFs. Activated TFs regulate gene expression involved in cell differentiation, proliferation, survival and migration. MAPK is also shown to participate in chromatin remodeling and nuclear import (Plotnikov *et al*., 2011; Rubinfeld and Seger, 2004; Yoon and Seger, 2006).

Scaffold proteins, such as kinase suppressor of Ras (KSR), connector enhancer of KSR (CNK) and β-arrestin, play a critical role in the sequential activation of kinases (Dhanasekaran *et al*., 2007). They can form a multiprotein complex with 2 or more components (e.g. ERK, MAPK and sometimes Raf) and participate in the Ras-MAPK signaling pathway, which concentrates multiple kinases in an adjacent area, facilitates kinase activity and the sequential activation to accelerate signal transmission (Claperon and Therrien, 2007; Dard and Peter, 2006; Dhanasekaran *et al*., 2007; Udell *et al*., 2011; Yao and Seger, 2009; Yoon and Seger, 2006).

In summary, the Ras-MAPK pathway is a cascade process that requires plenty of adaptor proteins and kinases (Figure 1.7). Initially, Shc is recruited to the phosphotyrosine residue of activated RTK via its PTB domain. RTKs phosphorylate tyrosine residues in Shc. Grb2 can bind to the phosphotyrosine residue in either RTK or Shc through its SH2 domain and translocate to plasma membrane. Grb2-associated Sos activates membrane-bound Ras by converting the GDP-bound state to the GTP bound state. Activated Ras results in sequential

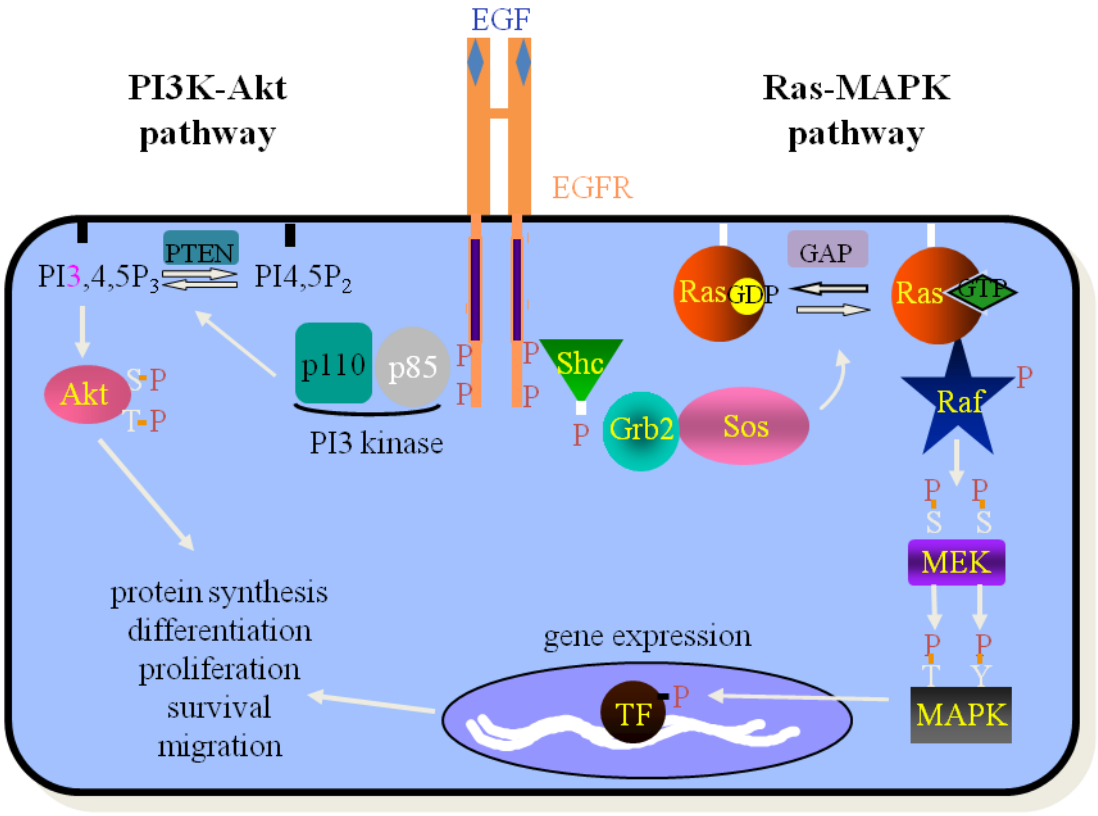

Figure 1.7 Selected RTK-activated signaling pathways. When growth factors bind to RTKs, the receptors are phosphorylated and recruit many intracellular signaling molecules. Several signaling pathways are then activated. For the Ras-MAPK pathway, Shc is recruited to the phosphotyrosine residue of activated RTK, which phosphorylates tyrosine residues in Shc. Grab2 can bind to the phosphotyrosine residue in either RTK or Shc and translocate to the plasma membrane. Grb2-associated Sos activates membrane-bound Ras by converting the GDP-bound state to the GTP-bound state. GAP negatively regulates Ras. Activated Ras results in sequential activation of kinases (Raf, MEK and MAPK). The series of activated kinases are involved in regulation of gene expression that affects cellular functions. For the PI3K-Akt pathway, activation of PI3K by RTK catalyzes the phosphorylation of $PI4,5P_2$ to $PI3,4,5P_3$ and thereby recruits Akt to the plasma membrane. Akt is subsequently activated and phosphorylates a number of intracellular proteins involved in different cellular processes. PTEN reverses PI3K-induced lipid phosphorylation and switches off the PI3K-Akt pathway.

activation of kinases (Raf, MEK and MAPK). The series of activated kinases are involved in regulation of gene expression that affects many cellular functions.

1.3.2 The Phosphatidylinositol 3 Kinase-Akt Signaling Pathway

PI3K is a member of an intracellular lipid kinase family that phosphorylates the 3-position of the inositol ring of phosphatidylinositol and yields lipid second messengers. Based on the structure and function, it is divided into 3 classes: class I, class II and class III (Damilano *et al*., 2010; Geering *et al*., 2007a; Ghigo *et al*., 2011). The best characterized class I PI3K preferentially takes part in converting phosphatidylinositol 4,5 bisphosphate ($PI4,5P_2$) to phosphatidylinositol 3,4,5 trisphosphate ($PI3,4,5P_3$). Depending on the receptor that activates class I PI3K, it is further divided into class IA PI3K that can be activated by RTKs and class IB PI3K that can be activated by GPCRs (Donato *et al*., 2010; Hawkins *et al*., 2006). Class IA PI3K is a heterodimer consisting of a regulatory subunit (p85α, p55α, p50α, p85β or p55γ) and a p110 catalytic subunit (p110α, p110β or p110δ) (Okkenhaug and Vanhaesebroeck, 2001; Ward *et al*., 2011).

p85α is the best studied among 5 regulatory subunit isoforms of class IA PI3K. The N-terminal SH3 domain can bind to P-X-X-P sequences in proline-rich proteins (Figure 1.8). p85 monomers can form dimers via their own SH3 domain and the first proline-rich region. The following breakpoint cluster region homology (BH) domain can bind to GTPases (Fry and Waterfield, 1993) and has GAP activity (Chamberlain *et al*., 2004). The 2 proline-rich regions can interact with many proteins including its SH3 domain. In the C-terminus, a p110 binding domain is flanked by 2 SH2 domains that are responsible for binding to tyrosine-phosphorylated proteins containing pY-X-X-M motif such as activated RTKs. Both the nSH2 and p110 binding domain are involved in p110 binding (Backer, 2010; Geering *et al*., 2007a; Krasilnikov, 2000; Wu *et al*., 2007). p85β shares the same domain structure as p85α. However, p55α, p50α and p55γ isoforms lack the N-terminal SH3 and BH domains and have different unique amino acid sequences in their N-terminus (Hawkins *et al*., 2006; Okkenhaug and Vanhaesebroeck, 2001).

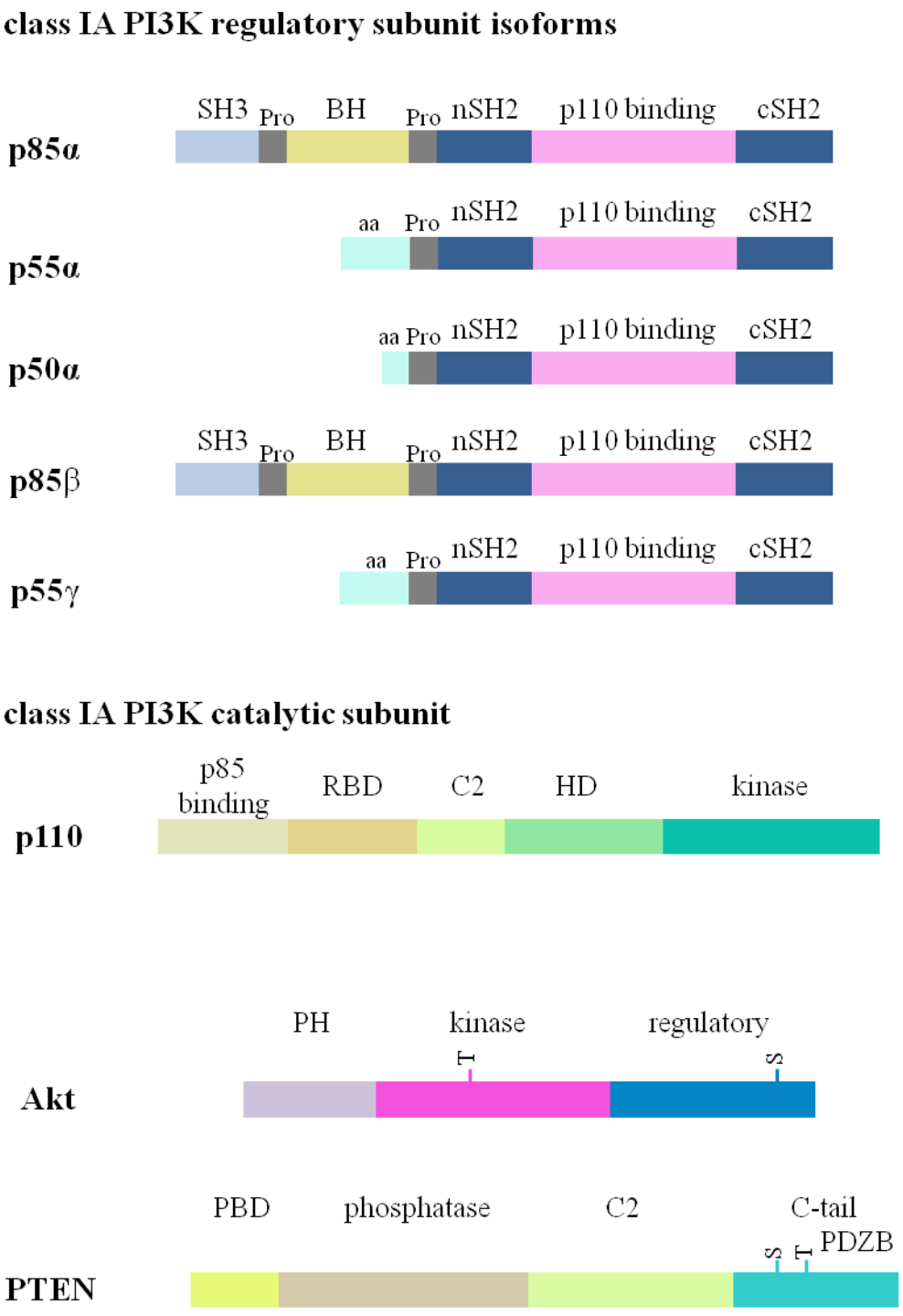

Figure 1.8 The domain structures of several intracellular signaling proteins involved in the PI3K-Akt pathway. These proteins are class IA PI3K, Akt and PTEN.

The p110 catalytic subunit isoforms have similar structures. They all consist of a p85 binding domain, a RBD domain, a protein kinase C homology 2 (C2) domain that has affinity for membrane, a helical domain (HD) involved in scaffold structure and a kinase domain (Figure 1.8) (Berndt *et al*., 2010; Zhao and Vogt, 2008). The N-terminal p85 binding domain interacts with the p110 binding domain in p85, which contributes to the dimerization between p85 and p110 and thus stabilizes and inhibits p110. Upon RTK activation, p85 docking to RTKs induces conformational change, relieves the inhibition of p110 by p85 and activates p110 catalytic activity. As well, p85 binding to RTKs translocates PI3K to the plasma membrane to facilitate the approach to $PI4,5P_2$ by p110 (Backer, 2010; Fu *et al*., 2004; Geering et al., 2007b; Vadas *et al*., 2011; Yu *et al*., 1998). The RBD domain binding to GTP-bound Ras can also promote p110 kinase activity, which links PI3K activation with the Ras-MAPK pathway (Hawkins *et al*., 2006). The activated PI3K catalyzes the phosphorylation of $PI4,5P_2$ to $PI3,4,5P_3$, which provides binding sites for proteins containing PH domain, such as Akt and phosphoinositide-dependent kinase (PDK) (Donato *et al*., 2010; Song *et al*., 2005; Vadas *et al*., 2011).

Akt, also called protein kinase B (PKB), is a 57 kDa serine/threonine kinase consisting of 3 isoforms: Akt1, Akt2 and Akt3. They all share the same structure with the kinase domain flanked by a PH domain and a regulatory domain (Figure 1.8). Akt binding to $PI3,4,5P_3$ through its N-terminal PH domain localizes Akt to the plasma membrane and causes a conformational change to expose phosphorylation residues (Fresno Vara *et al*., 2004; Osaki *et al*., 2004; Scheid and Woodgett, 2003; West *et al*., 2002). Akt can be phosphorylated at a threonine residue (Thr308 in Akt1, Thr309 in Akt2, Thr305 in Akt3) in the activation loop by PDK1 and a serine residue (Ser473 in Akt1, Ser474 in Akt2, Ser472 in Akt3) in the C-terminus to become fully activated (Chang *et al*., 2003a; Nicholson and Anderson, 2002; Osaki *et al*., 2004; Song *et al*., 2005). The kinase that phosphorylates the serine residue was unclear for a long time and recent research shows that mammalian target of rapamycin complex 2 (mTORC2) is the predominant kinase responsible for the serine residue phosphorylation (Hay, 2011; Hers *et al*., 2011).

Activated Akt can translocate into the cytoplasm or the nucleus and phosphorylate various substrates containing R-X-R-X-X-S/T-ψ sequences (ψ is a bulky hydrophobic residue), such as the Bcl-2/Bcl-X antagonist of cell death (BAD), caspase-9 and glycogen synthase kinase (GSK) 3 (Hanada *et al*., 2004; Hers *et al*., 2011). Akt1 is widely distributed and can regulate cellular processes including protein synthesis, cell cycle control, apoptosis, proliferation and survival (Chang *et al*., 2003a; Kandel and Hay, 1999). Akt2 plays a role in insulin signaling, while Akt3 functions in brain development (Almhanna *et al*., 2011; Chaanine and Hajjar, 2011).

Phosphatase and tensin homolog deleted on chromosome 10 (PTEN) is a 47 kDa dual lipid/protein phosphatase that mainly functions in dephosphorylation of $PI3,4,5P_3$ to $PI4,5P_2$ (Blanco-Aparicio *et al*., 2007; Knobbe *et al*., 2002). It functions as a tumor suppressor by antagonizing PI3K and negatively regulating PI3K-Akt pathway (Simpson and Parsons, 2001; Yin and Shen, 2008). Its $PI4,5P_2$ binding domain (PBD), phosphatase domain and C2 domain are responsible for lipid binding and both phosphatase and C2 domains confer the catalytic activity (Figure 1.8). There are multiple threonine and serine residues for phosphorylation and a PSD-95/Dlg/ZO-1 binding (PDZB) motif (Leslie and Downes, 2004; Steelman *et al*., 2004; Tamguney and Stokoe, 2007). These phosphorylated residues can interact with the phosphatase and C2 domains to stabilize PTEN and block membrane localization, playing a regulatory role. Dephosphorylation of these residues releases the C-terminal tail from these functional domains and allows plasma membrane localization via newly exposed basic residues in the phosphatase and C2 domains, as well as the PBD (Maehama *et al*., 2004). The PDZB motif binds PDZ-containing scaffold proteins such as Na^+/H^+ exchanger regulatory factor (NHERF) and membrane-associated guanylate kinase with inverted organization (MAGI) proteins that further localize PTEN to sites of RTK-associated PI3K lipid products (Bonifant *et al*., 2007; Mellor *et al*., 2012). PTEN is also a weak phosphatase towards some proteins including itself though the significance of these protein targets is unclear (Knobbe *et al*., 2002; Tamguney and Stokoe, 2007).

In summary, the PI3K-Akt pathway starts by the p85 regulatory subunit binding to

activated RTKs via SH2 domains. This activates the p110 catalytic subunit and localizes PI3K to the plasma membrane. Then PI3K catalyzes the phosphorylation of $PI4,5P_2$ to $PI3,4,5P_3$ and thereby recruits Akt to the plasma membrane. Akt is subsequently activated and phosphorylates a number of intracellular proteins involved in different cellular processes. PTEN reverses PI3K-induced lipid phosphorylation and switches off the PI3K-Akt pathway (Figure 1.7).

1.4 Receptor-Mediated Endocytosis

Endocytosis is a cellular process in which a variety of molecules are taken up from the cell surface into cells (Scita and Di Fiore, 2010). There are different methods to perform the internalization, such as phagocytosis, pinocytosis, clathrin-dependent endocytosis and clathrin-independent endocytosis (Sahay *et al*., 2010; Zaki and Tirelli, 2010). They are involved in several processes including extracellular nutrients uptake, synaptic vesicle recycling, cell surface receptor expression regulation, plasma membrane remodeling and cell polarity maintenance (Mukherjee *et al*., 1997). After internalization, the majority of molecules are recycled back to the cell surface or degraded in lysosomes. However, some molecules may traffic to organelles such as Golgi and endoplasmic reticulum (Sigismund *et al*., 2012).

Receptor-mediated endocytosis of RTK plays an important role in normal physiological functions since continuously activated RTKs and signaling pathways are usually correlated with cancers and other diseases. Receptor-mediated endocytosis is ligand induced and regulates signaling pathways by limiting the numbers of RTKs for activation (Sorkin and von Zastrow, 2009). It removes activated RTKs from the plasma membrane and regulates RTKs accessibility to the plasma membrane and their ligands. It also disassociates ligands from RTKs and dephosphorylates RTKs to downregulate signal transduction. Some RTKs are even degraded in lysosomes (McMahon and Boucrot, 2011; Sigismund *et al*., 2012; Sorkin and Von Zastrow, 2002).

Clathrin-mediated receptor endocytosis is the best studied and most widely used mechanism for RTK internalization (Takei and Haucke, 2001). It occurs at specific sites with

clathrin-coated pits. Clathrin has 3 heavy chains and 3 light chains that are organized in a triskelion structure. Clathrin forms lattice-like clathrin-coated pit around the inner membrane, which is mediated by adaptor proteins (e.g. AP-2). AP-2 has a phospholipid-binding motif that preferentially binds to $PI4,5P_2$ on the plasma membrane (Mousavi *et al*., 2004; Sorkin, 2004; Zaki and Tirelli, 2010). It also facilitates activated RTKs clustering with clathrin. Clathrin does not bind to RTKs or the plasma membrane directly. The clathrin polymerization by AP-2 triggers invagination and budding of clathrin-coated pits (Boettner *et al*., 2012; Scita and Di Fiore, 2010; Sigismund *et al*., 2012). The mature vesicles are eventually cleaved from cell membrane by dynamin at the neck. Dynamin is a GTPase with a PH domain that can bind to $PI4,5P_2$. The polymerization of dynamin and GTP hydrolysis promote the vesicle scission. Once the vesicles are free in cytoplasm, the clathrin coat is disassembled from the vesicles and the components are recycled. The uncoated vesicles are able to tether to and fuse with early/sorting endosomes, which is mediated by the Rab5 GTPase (McMahon and Boucrot, 2011; Takei and Haucke, 2001; Ungewickell and Hinrichsen, 2007).

Caveolae-mediated receptor endocytosis is the most common clathrin-independent mechanism. Caveolin is a small flask-shaped scaffold protein that can oligomerize to form lipid rafts with the invaginating part of the plasma membrane (Nabi and Le, 2003; Parton and Richards, 2003). Like clathrin-mediated endocytosis, the scission of budding vesicles from the plasma membrane is also dynamin dependent and followed by vesicle uncoating, tethering and fusion (Doherty and McMahon, 2009; Sandvig *et al*., 2011; Sverdlov *et al*., 2007).

Rab proteins are a family of monomeric small Ras-related GTPases that cycle between active GTP-bound forms and inactive GDP-bound forms. There are more than 60 Rabs that have been identified and many of them are widely expressed (Pfeffer, 2001; Seabra *et al*., 2002). They participate in intracellular vesicle transport and determine the specificity during vesicle fusion and sorting in endocytosis by recruiting certain effectors to specific membranes (Hammer and Wu, 2002; Jordens *et al*., 2005; Stenmark, 2009). The post-translational isoprenylation modification of Rabs in the C-terminus allows for membrane binding

(Pereira-Leal *et al*., 2001). GDP-bound Rabs are usually present in cytoplasm and associate with guanine-nucleotide-dissociation inhibitors (GDIs) to bind the isoprenyl group to remove the Rab from a membrane. GDIs inhibit GDP release and stabilize the inactive form. When GDI-displacement factors (GDFs) target to Rab-GDP-GDI complexes, GDIs are released. This causes conformational change and exposes the prenyl group and allows Rab membrane localization to a specific vesicle. GEFs and GAPs respectively regulate the activation and deactivation processes of Rabs, much as they do for Ras proteins. Active GTP-bound Rabs recruit the specific effectors for membrane trafficking. GDIs can bind to and extract the inactive GDP-bound Rabs from the membrane (Goody *et al*., 2005; Horgan and McCaffrey, 2011; Seabra and Wasmeier, 2004).

The internalized vesicles containing RTKs fuse with the early/sorting endosomes in a Rab5-mediated process. Early endosome antigen 1 (EEA1) can bind to active Rab5 and phosphatidylinositol 3-phosphate (PI3P) on different endosomes, and thus tether the 2 endosomes together. The soluble N-ethylmaleimide sensitive factor attachment receptor (SNARE) complex formed by EEA1 tethering promotes the fusion event. As well, Rab5 can bind to phosphatases that can yield PI3P from $PI3,4,5P_3$ (Neel *et al*., 2005; Pfeffer, 2001; Stenmark, 2009; Teis and Huber, 2003).

When receptors are transported to the early/sorting endosomes, ligands are detached and receptors are dephosphorylated and deactivated. There are 3 possible pathways for sorting RTKs from early/sorting endosomes (Figure 1.9) (Scita and Di Fiore, 2010; Sorkin and Goh, 2008). The detailed mechanism is not fully understood yet. Some receptors are rapidly recycled back to the plasma membrane, which is regulated by Rab4. Some receptors are slowly recycled back to the plasma membrane via intermediate recycling endosomes, which is regulated by Rab4 and Rab11 (Neel *et al*., 2005; Platta and Stenmark, 2011; Sadowski *et al*., 2009). Some receptors are sorted to be degraded via the late endosomes and lysosomes, which is regulated by Rab7. Only a small fraction of receptors which are monoubiquitinated may be diverted into multivesicular bodies (MVBs) by endosomal sorting complexes required for transport (ESCRTs) that are linked to the

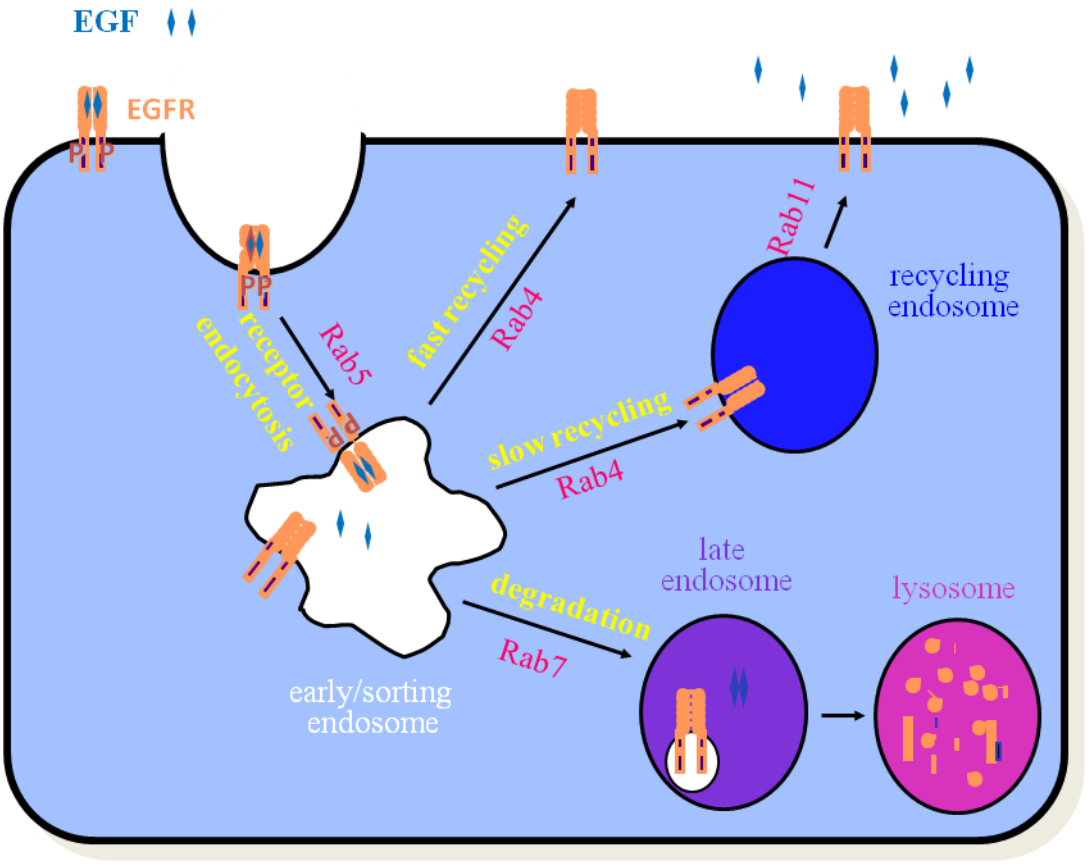

Figure 1.9 Receptor-mediated endocytosis. Activated RTKs are rapidly endocytosed via clathrin-dependent or clathrin-independent manner and transported to the early endosomes for sorting. Some receptors are dephosphorylated and rapidly recycled back to the plasma membrane. Some receptors are dephosphorylated and slowly recycled back to the plasma membrane via the intermediate recycling endosomes. Some receptors are sorted to be degraded via the late endosomes and lysosomes. Rab GTPases regulate vesicle fusion and sorting.

endosomal membrane. Mature MVBs within the late endosomes are then delivered to the lysosomes for degradation. The ubiquitination of receptors is induced by E3 ubiquitin ligases in the early steps of endocytosis (Jordens *et al*., 2005; Seabra *et al*., 2002; Sorkin and von Zastrow, 2009; Teis and Huber, 2003).

1.5 Ankyrins

Ankyrin is a family of intracellular adaptor proteins that associates with integral membrane proteins and cytoskeleton proteins and acts as a linker between plasma membrane proteins and the cytoskeleton. It can translocate and anchor membrane proteins, thus stabilizing them by providing structural support (Mohler, 2006; Rubtsov and Lopina, 2000). It has 3 members: ankyrin1 (ankyrin R, R for restricted expression), ankyrin2 (ankyrin B, B for broad expression) and ankyrin3 (ankyrin G, G for general expression). Ankyrin1 is found in erythrocyte, brain, cardiac muscle and skeletal muscle, and mainly in erythrocyte. Ankyrin2 is expressed in brain, heart and thymus. Ankyrin3 is expressed in brain, heart, kidney, lung, liver, intestine, skeletal muscle and epidermis. However, ankyrin2 and ankyrin3 are primarily expressed in heart (Cunha and Mohler, 2006; Curran and Mohler, 2011). These members all have several isoforms based on the alternative splicing of genes encoding for ankyrin (Hashemi *et al*., 2009).

Ankyrin1, ankyrin2 and ankyrin3 share the same domain structure that consists of an N-terminal membrane protein-binding domain, a spectrin-binding domain, a death domain and a C-terminal regulatory domain (Figure 1.10) (Bennett and Healy, 2008; Rubtsov and Lopina, 2000). The membrane protein-binding domain has 24 ankyrin repeats that each consists of 33 amino acids and 2 α-helices linked by β-hairpin loop. These ankyrin repeats are organized in a superhelical spiral structure and mediate interactions with membrane proteins in a multivalent manner (Cunha and Mohler, 2011; Czogalla and Sikorski, 2010; Ignatiuk *et al*., 2006; Mohler, 2006). The spectrin-binding domain can bind to β-spectrin in the cytoskeleton and thus links membrane proteins to the cytoskeleton (Hashemi *et al*., 2009). The role of death domain is still

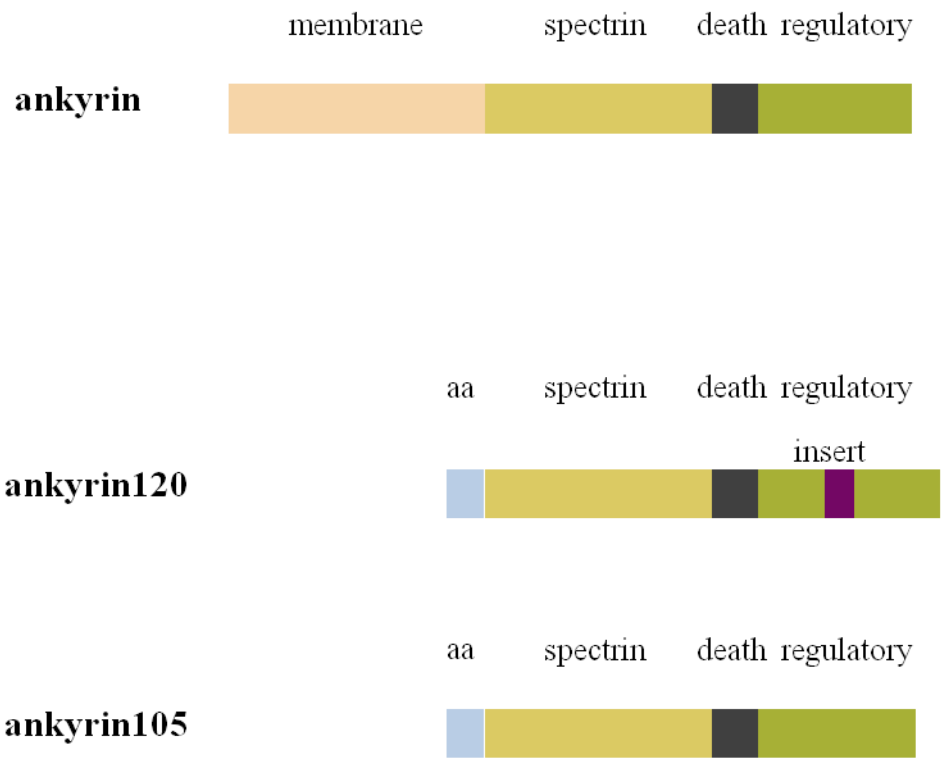

Figure 1.10 The domain structure of ankyrin. Ankyrin consists of an N-terminal membrane protein-binding domain, a spectrin-binding domain, a death domain and a C-terminal regulatory domain. The smaller isoforms of ankyrin3 (ankyrin120 and ankyrin105) lack the membrane protein-binding domain, but have 6 unique amino acids in the N-terminus. Ankyrin120 has an insert in its regulatory domain.

unclear, but it has been shown that death domain of ankyrin3 in renal tubules is related to cell death. It is the most highly conserved in sequence and may function together with regulatory domain (Bennett and Healy, 2009; Mohler, 2006). The regulatory domain is the most divergent among all 3 members. It controls the affinity of ankyrin binding to specific membrane proteins and spectrin (Cunha and Mohler, 2006; Lambert and Bennett, 1993).

Ankyrin3 is the most recently characterized and the most widely distributed. It is expressed in different isoforms of 480 kDa, 270 kDa, 215 kDa, 200 kDa, 190 kDa, 170 kDa and 100-120 kDa depending on cell type (De Matteis and Morrow, 1998; Ignatiuk *et al*., 2006; Rubtsov and Lopina, 2000). Some isoforms have inserts between the membrane protein-binding domain and the spectrin-binding domain and/or within the regulatory domain. The larger isoforms containing all domains are plasma membrane-associated proteins. The smaller isoforms (e.g. ankyrin120 and ankyrin105) lack the N-terminal membrane protein-binding domain and are localized to late endosomes and lysosomes (Figure 1.10) (Ignatiuk *et al*., 2006). They have 6 unique amino acids in the N-terminus. In addition, ankyrin120 has an insert in its regulatory domain (Hoock *et al*., 1997; Peters *et al*., 1995). Our laboratory has previously used a phage display library to select hexapeptides that could bind to the SH2 domain of the PI3K regulatory subunit p85 (King *et al.*, 2000). Ankyrin3 was considered as a possible p85-binding protein due to the presence of the hexapeptides that can bind to p85 SH2 domain. Subsequently, our laboratory has shown that p85 directly binds to the smaller isoforms (ankyrin120 and ankyrin105) of ankyrin3 and the binding requires the spectrin-binding domain and the regulatory domain of ankyrin3, and p110-binding domain and cSH2 domain of p85 (Ignatiuk *et al.*, 2006). Overexpression of ankyrin120 and ankyrin105 promotes lysosomal-mediated degradation of the PDGFR in NIH 3T3 cells and differentially affects PDGFR downstream signaling pathways (Ignatiuk *et al*., 2006). These results suggest that the smaller isoforms of ankyrin3 might facilitate RTK degradation and downstream signaling pathway downregulation.

1.6 Cancer Therapeutics to Downregulate ErbB2

Breast cancer is the most common cancer in women and the second leading cancer that causes death (Howard and Bland, 2012). Statistics suggests that about 200,000 cases of breast cancer occur every year in the United States, which is about 30% of the annual new cancer cases (Dean-Colomb and Esteva, 2008; Engel and Kaklamani, 2007). It is predicted that the morbidity in women in their lifetime risk is 1 in 8 (Downs-Holmes and Silverman, 2011). Breast cancer is identified as a heterogeneous disease including 5 major subtypes by studying the gene expression profiling using DNA microarrays (Van der Auwera *et al*., 2010). The classification of subtypes is based on estrogen receptor (ER), progesterone receptor (PR) and ErbB2 status (Nguyen *et al*., 2008). According to ER expression, there are 2 categories: ER-positive tumors (luminal A and luminal B subtypes) and ER-negative tumors (ErbB2-positive, basal-like and normal-like subtypes) (Bertucci *et al*., 2012; Geyer *et al*., 2012). Luminal A (ER+ or PR+ and ErbB2-) and luminal B (ER+ or PR+ and ErbB2+) subtypes have similar expression profiles in luminal mammary epithelial cells. But they have different expression of ER-related genes and proliferative genes, giving rise to different survival rates (Bertucci *et al*., 2012; Nguyen *et al*., 2008). ER-negative tumors have relatively low expression of ER and PR and are usually associated with poor prognosis, high recurrence rate and low survival rate compared to ER-positive tumors (Toft and Cryns, 2011). ErbB2-positive (ER- or PR- and ErbB2+) subtype is more aggressive and worse prognosis than ErbB2-negative subtypes (Mukai, 2010). It is shown that about 20% to 30% of breast cancer results from ErbB2 overexpression (Pedersen *et al*., 2008). Basal-like and normal-like tumors substantially overlap with triple-negative (ER-, PR- and ErbB2-) breast cancer (Carey *et al*., 2010; Pal *et al*., 2011). Basal-like tumors take up 15% to 25% of breast cancer cases (Rakha and Ellis, 2009). The study of different subtypes may help to improve the understanding of clinical characteristics of breast cancer and develop new strategies for prognosis and therapeutic treatment (Espinosa *et al*., 2011; Finnegan and Carey, 2007).

ErbB2 has a constitutively exposed dimerization arm, which can homodimerize and heterodimerize with other members of the EGFR subfamily without ligand docking (Burgess *et al*., 2003). ErbB2 is internalization resistant, so ErbB2-containing dimers may have enhanced and prolonged activation of receptors and downstream signaling pathways (Hommelgaard *et al*., 2004). Overexpression of ErbB2 is usually linked to cancers, especially breast cancer (Pedersen *et al*., 2008). Therefore, ErbB2 has been characterized as a viable target for cancer therapeutics. Many drugs that can directly target to ErbB2 or induce ErbB2 downregulation have been developed due to the increasing understanding of ErbB2 (Higgins and Baselga, 2011; Sachdev and Jahanzeb, 2012).

1.6.1 Geldanamycin

Geldanamycin (GA) and its analog 17-allylamino-17-demethoxygeldanamycin (17-AAG) are benzoquinone ansamycin antibiotics that can inhibit heat shock protein (Hsp) 90 chaperone function (Austin *et al*., 2004; Neckers *et al*., 1999). Hsp90 is widely expressed, but at high levels in cancer cells. It can bind to multiple client proteins including ErbB2, p53 and Akt, and stabilize them. Hsp90 has an N-terminal ATPase domain that has a conserved ATP/ADP-binding pocket. Hsp90 cycles between 2 conformations based on ATP or ADP binding (Chiosis *et al*., 2004; Hao *et al*., 2010). In the ADP-bound form, client proteins bind to Hsp90, which is mediated by the Hsp40/Hsp70 complex and the Hsp40/Hsp70 organizing protein (HOP). In the ATP-bound form, Hsp40/Hsp70 and HOP are released and the mature complex with client protein is formed (Fukuyo *et al*., 2010). Geldanamycin competitively binds to the ATP/ADP-binding pocket and then Hsp90 mimics the ADP-bound form. The associated Hsp70 recruits the E3 ligase heat shock cognate protein 70-interacting protein (HIP) and the client protein is ubiquitinated and sorted for degradation via the proteasome (Blagosklonny, 2002; Neckers, 2002; Pedersen *et al*., 2009). It has been demonstrated that geldanamycin promotes ErbB2 downregulation from the plasma membrane, but the mechanism and whether it is via a clathrin-dependent or proteasome-dependent manner is still controversial (Barr *et al*.,

2008; Lerdrup *et al*., 2006; Pedersen *et al*., 2008). Although its anti-cancer potential has been confirmed *in vitro*, geldanamycin is still not used in clinical treatment because it has severe hepatotoxicity in animal models when using a therapeutically effective dose (Supko *et al*., 1995). In addition, it is not soluble in water and not metabolically stable, which suggests that it is not a good candidate for drug development. Different geldanamycin derivatives have been developed to minimize these limitations (Fukuyo *et al*., 2010; Le Brazidec *et al*., 2004).

1.6.2 Herceptin

Nowadays, a variety of molecules that can specifically bind to the extracellular or intracellular domain of ErbB2 have been identified as drugs for cancer therapy. Monoclonal antibodies or ligand antagonists have been used to bind to the extracellular domain, whereas the binding to the intracellular domain is performed by tyrosine kinase inhibitors (TKIs) (Saxena and Dwivedi, 2012).

Herceptin (Trastuzumab) is a humanized monoclonal antibody against ErbB2 extracellular domain IV. It is the first drug that can target to ErbB2 that was approved by the FDA and has been used in clinical treatment for ErbB2-positive cancer for more than 10 years (Pegram and Liao, 2012). It has 2 antigen-specific binding sites. The targeting of herceptin to extracellular domain of ErbB2 blocks ErbB2-induced dimerization and inhibits intracellular tyrosine kinase activity (Hudis, 2007). It can facilitate ErbB2 internalization and degradation, interrupt intracellular downstream signaling pathways and prevent tumorigenesis (Sachdev and Jahanzeb, 2012; Spector and Blackwell, 2009). However, herceptin has some side effects such as cardiac toxicity, neutropenia and anemia (Chen *et al*., 2011; Chien and Rugo, 2010). In addition, some patients have inherent or acquired resistance to herceptin that may lead to relapse. The resistance results from other molecules that can interact with ErbB2 (Arteaga *et al*., 2012). For example, blockage of ErbB2 may contribute to an increase in the expression of other EGFR members as a compensating response (Arpino *et al*., 2007). PTEN loss, PI3K mutation and abnormality of the extracellular domain of ErbB2 are the mechanisms that have been shown to contribute to herceptin resistance (Dean-Colomb and Esteva, 2008; Mukai, 2010).

2.0 RATIONAL AND OBJECTIVES

Our laboratory has previously shown that the PI3K regulatory subunit p85 directly binds to the smaller isoforms (ankyrin120 and ankyrin105) of ankyrin3. Overexpression of ankyrin120 and ankyrin105 promotes lysosomal-mediated degradation of the PDGFR in NIH 3T3 cells and differentially affects PDGFR downstream signaling pathways (Ignatiuk *et al*., 2006). These results suggest that the smaller isoforms of ankyrin3 might facilitate RTK degradation and downstream signaling pathway downregulation. To determine whether ankyrin105 can induce degradation of multiple RTKs similarly, we extended these studies to EGFR and its downstream signaling in this project.

2.1 Hypothesis

Ankyrin105 facilitates degradation of EGFR and enhances the downregulation of signaling pathways.

2.2 Specific Objectives

1. Determine effect of ankyrin105 expression on EGFR levels and activation state.
2. Characterize downstream EGFR signaling in selected ankyrin105 expression cells.

3.0 MATERIALS AND METHODS

3.1 Materials

3.1.1 Cell Lines and Tissue Culture Conditions

Five cell lines were selected for this thesis, which were COS-1, HEK293T, MCF10A, MDA-MB-231 and AU565, respectively. All cell lines were purchased from American Type Culture Collection (ATCC), except for HEK293T cell, which was received from Dr. Roesler' s laboratory (Department of Biochemistry, University of Saskatchewan). Detailed information and features of each cell line are shown in Table 3.1. EGFR and ErbB2 levels of three breast cell lines are shown in Table 3.2. Cell culture media and condition are shown in Table 3.3. MCF10A cells were detached from the plates by TrypLE Express (Gibco 12605) and cells from all other cell lines were trypsinized by Trypsin-EDTA (ethylene diamine tetraacetic acid) (Gibco 15400).

Special safety precautions were used when working with cholera toxin. Biosafety Level 2 rules in the Biosafety Manual and Hazardous Waste Disposal Manual (from Department of Health, Safety and Environment, University of Saskatchewan) were followed. Biohazard signs were placed on the biological safety cabinet (BSC) and incubator. All the experiments that required cholera toxin were done in BSC. Two pairs of gloves and a disposable lab coat were worn and disposable plasticware were used when handling cholera toxin. Before the experiment, bench coat was placed on the working area in BSC. After the experiment, waste solution was inactivated with 10% bleach for 1 hour prior to disposal. All other waste, including plates, tubes, plastic pipettes, plastic pipettes, gloves, etc., were decontaminated with 10% bleach for 1 hour, and then sealed in labeled double bags and sent for disposal. The BSC working area was also decontaminated with 10% bleach for 1 hour and then wiped down with distilled water and 70% ethanol.

Table 3.1 Selected cell lines and features

Cell Line	Catalogue Number	Cell Type	Feature
COS-1	CRL-1650	Simian Vacuolating Virus 40 (SV40) transformed African green monkey kidney fibroblast-like cell	express EGFR
HEK293T	N/A	SV40 transformed human embryonic kidney cell	express EGFR
MCF10A	CRL-10317	immortalized human breast epithelial cell, not tumorigenic or transformed	express EGFR
MDA-MB-231	HTB-26	human breast cancer epithelial cell	express EGFR
AU565	CRL-2351	human breast cancer epithelial cell	express EGFR and ErbB2

Table 3.2 Semiquantitative measurement of EGFR and ErbB2 levels in three human breast cell lines. EGFR and ErbB2 levels were measured and normalized from Western blots using Scion Image software. The data were taken from a protein file (Neve *et al*., 2006).

	MCF10A	MDA-MB-231	AU565
EGFR	821	5994	6085
ErbB2	705	1991	10750

Table 3.3 Culture media and growth conditions of cell lines used

Cell Line	Cell Culture Media	Cell Culture Conditions
COS-1	Dulbecco's Modified Eagle Medium (DMEM, Gibco 12100), 10% fetal bovine serum (FBS, PAA A15-237)	37ºC, 5% CO_2
HEK293T	Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F-12, Gibco 12500), 10% FBS	37ºC, 5% CO_2
MCF10A	advanced DMEM/F-12 (Gibco 12634), 5% horse serum (PAA B15-023), 10 μg/mL insulin (Sigma I1882), 10 ng/mL EGF (Sigma E9644), 0.5 μg/mL hydrocortisone (Sigma H0888), 1 mM L-glutamine (Sigma G2150), 0.1 μg/mL cholera toxin (Sigma C8052)	37ºC, 5% CO_2
MDA-MB-231	Leibovitz's L-15 Medium (Gibco 41300), 10% FBS	37ºC, 100% air
AU565	Roswell Park Memorial Institute (RPMI) Medium 1640 (Gibco 31800), 10% FBS, 10mM HEPES (Sigma H3375), 1 mM sodium pyruvate (Gibco 890-1840IM), 2.5 mg/mL D-glucose (BDH B10117)	37ºC, 5% CO_2

3.1.2 Plasmids and Adenovirus Vectors

pHA_3 plasmid encoding 3 copies of hemagglutinin (HA) (YPYDVPDYA) was constructed by Yun Fang, which was modified from pRc/CMV2 vector (Invitrogen) (Figure 3.1) (King *et al*., 2000). The vector has an SV40 replication origin, which would help the vector replicate in SV40 transformed cells, such as COS-1 and HEK293T cells (Ali and DeCaprio, 2001; Asano *et al*., 1985). The cytomegalovirus (CMV) promoter produces high expression of insert gene in a variety of mammalian cells. pHA_3 plasmid is used as a control for other recombinant plasmids modified from itself, which would be pHA_3-Ank105 in this thesis.

Recombinant pHA_3-Ank105 plasmid was generated by Josh Giroux. Ankyrin105 (amino acids 874-1588, 1783-1961) has spectrin-binding domain and regulatory domain but without insert A, B and C. Ankyrin105 DNA and pHA_3 vector were both digested by *Bgl*II and *Eco*RI. Then ankyrin105 DNA was subcloned into the vector. There were 6 unique amino acids (MALPHS) in the N-terminal region of ankyrin105, which would be expressed right after the HA tags in the resulting fusion protein HA-Ank105 (Ignatiuk *et al*., 2006).

pEGFP-C1 plasmid (BD Biosciences 6084-1) contains SV40 replication origin and CMV promoter, which would help to get high expression of inserted green fluorescent protein (GFP) gene (Figure 3.2). This plasmid was used to optimize the amount of plasmid DNA for calcium phosphate transfection in this thesis.

There were 2 adenoviral vectors used in the experiments. Adenovirus-CMV-GFP control (Ad-GFP) (Vector Biolabs 1060) was constructed using adenovirus-type 5 (dE1/E3) and expressed GFP. It had a titer of 1×10^{10} plaque forming units (PFU)/mL and was stored at -80ºC. It was commonly used as a control for other recombinant adenoviruses using the Ad-GFP viral backbone, which would be adenovirus-CMV-GFP+HA_3-Ank105 (Ad-GFP+HA-Ank105) in this thesis. The HA-Ank105 gene was cloned into a shuttle vector by Levi Furber. Then the vector was sent to Vector Biolabs for custom adenovirus construction service. The recombinant adenovirus Ad-GFP+HA-Ank105 used the Ad-GFP viral backbone and expressed both GFP

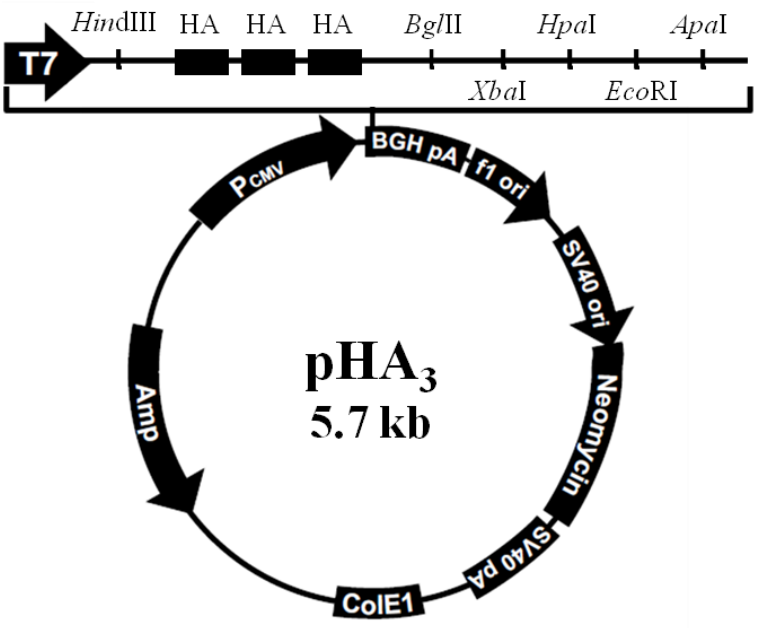

Figure 3.1 Map of pRc/CMV2 plasmid and modifications to generate pHA_3. The figure was modified from Invitrogen website (http://tools.invitrogen.com/content/sfs/manuals/prccmv2_man.pdf). This plasmid contains CMV promoter, T7 promoter, bovine growth hormone (BGH) polyadenylation signal, f1 origin, SV40 replication origin, neomycin resistance gene, SV40 polyadenylation signal, ColE1 origin, ampicillin resistance gene and multiple restriction sites for gene insertion. Three copies of HA were cloned into these multiple restriction sites.

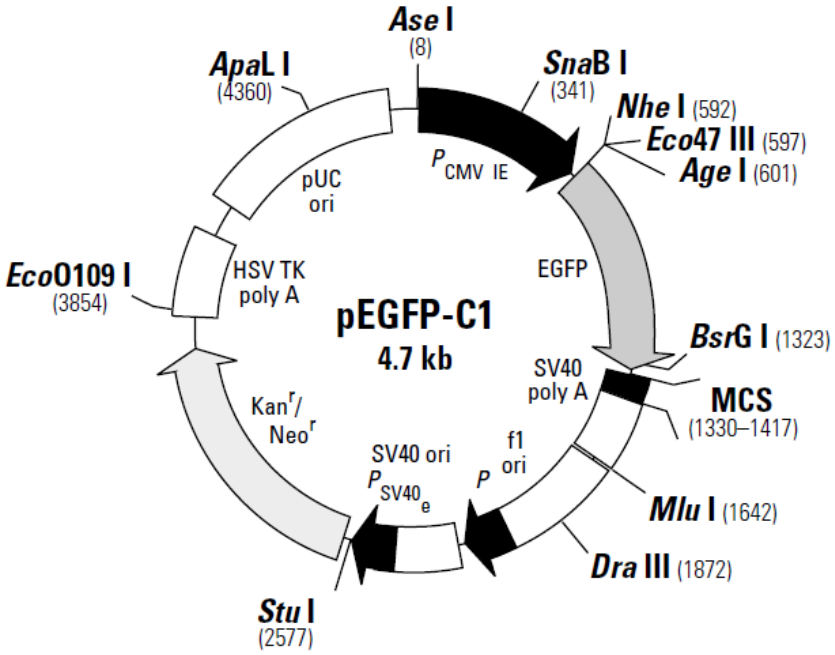

Figure 3.2 Map of pEGFP-C1 plasmid. The figure was taken from Molecular Info website (http://www.molecularinfo.com/MTM/K/K2/K2-1/pEGFPc1.pdf). This plasmid contains CMV promoter, GFP gene, SV40 polyadenylation signal, f1 origin, SV40 replication origin, SV40 promoter, kanamycin/neomycin resistance gene, herpes simplex virus (HSV) thymidine kinase (TK) polyadenylation signal, pUC replication origin and multiple restriction sites for gene insertion.

and HA-Ank105. GFP and HA-Ank105 genes were from the same transcript, but expressed as 2 separate proteins. Ad-GFP+HA-Ank105 had the titers of 6.2×10^{10} PFU/mL and 5.6×10^{10} PFU/mL (from different batches) and was stored at -80ºC.

3.1.3 Antibodies

Different antibodies were used in the experiments for either Western blot (WB) or flow cytometry (FCM). Detailed information and working conditions for these antibodies are listed in Table 3.4.

3.1.4 Other Reagents and Supplies

All chemicals and reagents used in the experiments were analytical grade or higher and were purchase from VWR, Sigma or BDH, unless otherwise indicated. The compositions of the buffers are listed in Table 3.5.

3.2 Cell Culture Techniques

3.2.1 Transient Transfection

3.2.1.1 COS-1 Cells Transfection

HA-Ank105 was introduced into COS-1 cells by transient transfection using Lipofectamine reagent (Invitrogen 18324). COS-1 cells were split 1:5 into 10 cm plates on day 1, which were then about 40% confluent by the next day. On day 2, 3 μg or 6 μg plasmid DNA encoding HA-Ank105 was added into 600 μL Opti-MEM Reduced Serum Media (Gibco 31985) and then combined with a mixture of 600 μL Opti-MEM Reduced Serum Media and 18 μL Lipofectamine reagent. After 15 minutes incubation at room temperature, 4.8 mL serum-free DMEM was added to the combined solution to make the transfection media. For each 10 cm plate, the cells were washed once with 5 mL warm serum-free DMEM and the 6 mL transfection media was added to the cells. The cells were incubated at 37ºC and 5% CO_2 for 5

Table 3.4 List of antibodies

Antibody	Company and Catalogue Number	Species	Working Condition
Ankyrin 3 (AP)	Dr. Anderson lab made	rabbit	WB: 1 μg/mL
EGFR (C74B9)	Cell Signaling, 2640	rabbit	WB: 1:1000
HER2/ErbB2 (29D8)	Cell Signaling, 2165	rabbit	WB: 1:1000
Phospho-Tyr (PY20)	Santa Cruz, sc-508	mouse	WB: 2 μg/mL
Akt	Cell Signaling, 9272	rabbit	WB: 1:1000
Phospho-Akt (Ser473) (pAkt)	Cell Signaling, 9271	rabbit	WB: 1:1000
ERK1 (MAPK)	BD, 610030	mouse	WB: 1:1000
Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (pMAPK)	Cell Signaling, 9101	rabbit	WB: 1:1000
GFP (FL)	Santa Cruz, sc-8334	rabbit	WB: 1 μg/mL
HA-probe (F7)	Santa Cruz, sc-7392	mouse	WB: 1 μg/mL
GAPDH	Ambion, AM4300	mouse	WB: 1 μg/mL
IRDye 680 Goat anti-Rabbit IgG	LI-COR, 926-32221	goat	WB: 1:10000
IRDye 680 Goat anti-Mouse IgG	LI-COR, 926-32220	goat	WB: 1:10000
IRDye 800CW Goat anti-Mouse IgG	LI-COR, 926-32210	goat	WB: 1:10000
Neu (9G6)	Santa Cruz, sc-08	mouse	FCM: 0.5 μg/1×10^6 cells
Goat anti-Mouse IgG_1-PE	Santa Cruz, sc-3764	goat	FCM: 0.5 μg/1×10^6 cells

Table 3.5 Buffer compositions

Buffer	Composition
Blocking buffer (for most Western blots)	5% (w/v) Carnation skim milk powder in PBS
Blocking buffer (for pTyr blots)	1% (w/v) BSA Fraction V in PBST
EGF dilution buffer	10 mM acetic acid, 2 mg/mL BSA
HBS (2×)	50 mM HEPES, 280 mM NaCl, 12 mM D-glucose, 10 mM KCl, 1.5 mM Na_2HPO_4, pH 6.94-6.943
Lower gel buffer	375 mM Tris-HCl, 0.1% (w/v) SDS, pH 8.8
PBS	137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4
PBST	0.1% (v/v) Tween-20 in PBS
SDS-PAGE running buffer	25 mM Tris, 192 mM glycine, 0.1% (w/v) SDS
SDS sample buffer	10% (w/v) glycerol, 5% (v/v) 2-mercaptoethanol, 2.3% (w/v) SDS, 0.0625 M Tris, 0.1% (w/v) bromophenol blue
Transfer buffer	47 mM Tris, 39 mM glycine, 3.75% (w/v) SDS, 20% (v/v) methanol
Upper gel buffer	125 mM Tris-HCl, 0.1% (w/v) SDS, pH 6.8

hours. An additional 6 mL of DMEM with 20% FBS was added to the plate without removing the transfection media and the cells were incubated at 37ºC and 5% CO_2 overnight. On day 3, the media was replaced by the standard media (DMEM with 10% FBS). On day 4, the cells were serum starved overnight (16 to 24 hours). An EGF stimulation experiment (see section 3.2.3 for detail) was carried out on day 5.

3.2.1.2 HEK293T Cells Transfection

HA-Ank105 was introduced into HEK293T cells by transient transfection using calcium phosphate. To optimize the amount of plasmid DNA used for transfection, HEK293T cells were split 1:5 into a 6-well plate in the morning of day 1. In the afternoon, different amounts (3 μg, 5 μg, 6.25 μg, 7 μg and 9 μg) of pEGFP-C1 plasmid were added to 120 μL 265 mM $CaCl_2$ and 125 μL 2×HBS. After 20 minutes incubation at room temperature, the mixture was added drop-wise to the cells with existing media. Then the cells were incubated at 37ºC and 5% CO_2 overnight. In the morning of day 2, the media was removed, and the cells were washed and fed with standard media (DMEM/F-12 with 10% FBS). To monitor GFP expression, the cells were photographed using a fluorescence microscope (Nikon TE300) on the third day.

Once the optimal amount of plasmid DNA was determined, plasmids encoding HA or HA-Ank105 were transfected into the cells. For each 10 cm plate, the transfection solution consisted of 30 μg pHA_3 or pHA_3-Ank105 plasmid DNA, 720 μL 265 mM $CaCl_2$ and 750 μL 2×HBS. The cells were transfected according to the protocol described above. In the afternoon of day 2, the cells were serum starved for 16 hours. An EGF stimulation experiment (see section 3.2.3 for detail) was carried out on day 3.

3.2.2 Transduction with Adenovirus Vector

In order to get high expression of ankyrin105 in MCF10A, MDA-MB-231 and AU565 cells, adenovirus transduction was used. According to the protocol from Vector Core (www.med.umich.edu/vcore), the cells should be about 50% confluent before the transduction

carried out. So we needed to optimize the number of cells plated to make sure the cells would be about 50% confluent for the next day. The cells from different cell lines were counted by Coulter Particle Count and Size Analyzer (Beckman Coulter Z2) and different numbers of cells were plated in 6-well plates. By observing the plates on the next day, we could decide the numbers of cells needed for 50% confluent in 6-well plates, which were listed in Table 3.6. The numbers of cells for 10 cm plates were calculated based on the relative surface areas.

Table 3.6 The numbers of cells for 50% confluent in 6-well plates and 10 cm plates

Cell Line	6-well Plate	10 cm Plate
MCF10A	7.5×10^4	4.42×10^5
MDA-MB-231	3×10^5	1.77×10^6
AU565	7.5×10^4	4.42×10^5

To determine how much adenovirus was required to obtain high protein expression, different multiplicities of infection (MOIs) were tested. The appropriate numbers of cells from 3 cell lines were plated in 6-well plates the day before transduction. The cells were about 50% confluent by the next day. The adenovirus was thawed on ice and 1:10 diluted in either PBS or cell culture media. The amount of adenovirus needed for a particular MOI was calculated from the following formula: Volume (μL) = (number of cells) × (desired MOI) / (PFU/μL). The different volumes of diluted adenovirus were added to cell culture media to make the total volume of 350 μL for each well. After aspirating existing media, the 350 μL of transduction media was added to each well and the cells were incubated at 37ºC for 1 hour. During the incubation, the plates were rocked every 10 to 15 minutes. Then the transduction media was replaced by the standard cell culture media and the cells were placed back to the incubator. The cells were observed using a fluorescence microscope each day up to 5 days post-transduction to monitor GFP expression in order to decide the optimal conditions.

For the transduction experiments performed in 10 cm plates in order to test ankyrin105 function, the one hour adenovirus incubation using the optimal MOI was carried out as mentioned above. The transduction media for each plate was 2 mL. The cells were serum starved 16 to 24 hours on the fourth day post-transduction and an EGF stimulation experiment (see section 3.2.3 for detail) was carried out on the fifth day.

Special safety precautions were used when working with adenovirus. Biosafety Level 2 rules in the Biosafety Manual and Hazardous Waste Disposal Manual were followed. The biohazard signs were placed on BSC and incubator. All the experiments that required adenovirus were done in BSC. Two pairs of gloves and a lab coat were worn when handling adenovirus. After the experiment, waste solution was inactivated with 10% bleach for 15 minutes and then discarded. The glass pipettes were soaked in 10% bleach for 15 minutes before sent for washing and sterilization. All other waste, including plates, tubes, plastic pipettes tips, gloves, etc., were decontaminated with 10% bleach for 15 minutes and then disposed in the biohazard bin. The BSC working area was also decontaminated with 10% bleach for 15 minutes and then wiped down with distilled water and 80% ethanol.

3.2.3 Growth Factor Stimulation

To characterize ankyrin105 function during the degradation of EGFR and downregulation of downstream signaling pathways in response to EGF stimulation, the cells were treated with EGF in a time course experiment. Before the experiment, the cells were about 80% to 90% confluent. The cells were serum starved for 16 to 24 hours using starving media (all other components for the standard cell culture media but with only 0.5% serum). Then the media was replaced by 3 mL stimulation media containing 80 nM EGF (diluted in EGF dilution buffer first) and the cells were incubated at 37ºC for indicated times in each experiment. Cells were lysed (see section 3.2.4 for detail) for further experiments.

3.2.4 Cell Lysate Preparation

After different treatments for various times, the cells were placed on ice and washed with cold PBS. For each 10 cm plate, 300 μL sodium dodecyl sulfate (SDS) sample buffer at 100ºC was used to collect the cells by scraping the plate. Then the cell lysates were boiled at 100ºC for 5 minutes. Because the lysates were viscous and hard to pipette an accurate volume, the cell lysates were sonicated for 5 seconds using the output control at 2 by Sonifier (Branson 450 P/S BIO) before use or storage at -20ºC or -80ºC. The protein concentration was obtained by Lowry Assay using Total Protein Kit, Micro Lowry, Peterson's Modification (Sigma TP0300).

3.3 Protein Analysis

3.3.1 Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The cell lysates containing total protein were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then followed by Western blot analysis (see section 3.3.2 for detail). SDS-PAGE was carried out in Mini-PROTEIN 4-Gel Electrophoresis System (Bio-Rad 165). 1 mm or 1.5 mm gels were cast between two glass plates and 10-well or 15-well combs were used depending on the volumes of loaded proteins. The SDS-PAGE gel was composed of resolving gel and stacking gel. Depending on the molecular weights of the detected proteins, different percentages (7.5%, 10% or 15% (w/v)) of acrylamide solution (29.2:0.8 acrylamide:bisacrylamide) in lower gel buffer (LGB) were used for resolving gels. 0.16% (w/v) ammonium persulfate (APS) and 0.1% (w/v) N,N,N',N'-tetramethylethylenediamine (TEMED) were added to facilitate gel polymerization. The stacking gel consisted of 4.5% acrylamide solution in upper gel buffer (UGB), 0.24% (w/v) APS and 0.1% (w/v) TEMED (Laemmli, 1970). After the gels polymerized, the Prestained Protein Ladder (Fermentas SM0671) and cell lysates containing the same amounts of total protein were loaded on the gels. The electrophoresis was performed in running buffer at a

constant voltage of 180 V for about 1 hour until the bromophenol blue went off the bottom of the gel.

3.3.2 Western Blot Analysis

The gel was soaked in transfer buffer for 15 minutes on the rocker at a low speed. During this time, one piece of nitrocellulose membrane (Whatman BA85 10401196) was soaked in distilled water and 6 pieces of 3 MM filter paper (Whatman 3030-700) were soaked in transfer buffer for each gel. Three pieces of 3 MM paper, the SDS-PAGE gel, nitrocellulose membrane and 3 pieces of 3 MM paper were placed in the Semi-Dry Blotting Apparatus (Fisher FB-SDB-2020) following the order from bottom to top. Each gel was transferred at a constant current of 450 mA for 30 minutes.

Nitrocellulose membrane was incubated in blocking buffer for 1 hour at room temperature or overnight at 4ºC on the rocker. Primary antibody, which was made in blocking buffer, was added and incubated for 1 hour at room temperature or overnight at 4ºC on the rocker. The blot was washed 4 times with PBST for 5 minutes each on the rocker. It was incubated in an infrared dye-labeled secondary antibody for 1 hour at room temperature or overnight at 4ºC on the rocker. The blot was washed 4 times with PBST for 5 minutes each again and rinsed in PBS to remove the tween. During the incubation with secondary antibody and the washing steps thereafter, the blot was covered with tinfoil to avoid the light. The blot was scanned using an Odyssey Infrared Imaging System (LI-COR 9201-01).

3.3.3 Flow Cytometry

Flow cytometry is a fluorescence-activated cell sorting technique to measure cell number, size and various properties in a flow system. The directly fluorescent-labeled cellular components or fluorescent-labeled antibodies against cellular proteins can be detected by the measuring the scattered light and fluorescent light (Bohn, 1980; Wilkerson, 2012). For the study of internalization of cell surface receptors in this thesis, flow cytometry was a very useful

approach for analyzing and quantifying cell surface ErbB2 levels.

Geldanamycin (EMD 345805) was dissolved in dimethyl sulfoxide (DMSO) to a concentration of 1 mM and stored at -20ºC. Herceptin (Roche) was dissolved in water to a concentration of 10 mg/mL and stored at -80ºC. AU565 cells were treated with 1 μM geldanamycin or 10 μg/mL herceptin for the indicated times in different experiments (see section 4.6.3 and 4.6.5 for detail).

After different treatments, the media was removed and the cells were washed once with PBS. The cells from each 10 cm plate (about 1×10^6 cells) were trypsinized by 0.05% Trypsin/EDTA/PBS and collected in 5 mL standard cell culture media. Then the cells were centrifuged for 5 minutes at $290\times g$ (Eppendorf 5810R) at 4ºC. The supernatant was aspirated and the pellet was resuspended in 100 μL flow cytometry staining buffer (eBioscience 00-4222) for blocking. After 15 minutes at 4ºC on the rocker, the cell suspension was centrifuged for 5 minutes at $371\times g$ (Eppendorf 5415R) at 4ºC. The pellet was resuspended in 100 μL staining buffer containing 0.5 μg Neu antibody (9G6). After 30 minutes at 4ºC on the rocker, the cell suspension was centrifuged for 5 minutes at $371\times g$ at 4ºC. The pellet was resuspended in 1 mL PBS to wash away any excess primary antibody. The washing process was repeated 3 times. The pellet was resuspended in 100 μL staining buffer containing 0.5 μg phycoerythrin (PE) -conjugated secondary antibody and incubated for 30 minutes at 4ºC on the rocker. The same steps were used to wash away any excess secondary antibody. During the incubation with secondary antibody and the washing steps thereafter, the cell suspension was covered with tinfoil to protect from light. The pellet was resuspended in 1 mL of 1% paraformaldehyde and could be stored at 4ºC for 1 week. Samples were analyzed by flow cytometry (Beckman Coulter Epics XL) using FlowJo software (version 8.8.6).

4.0 RESULTS

4.1 Ankyrin3, EGFR and ErbB2 Expression in Selected Cell Lines

It has been shown that overexpression of the small isoforms of ankyrin3 (120 kDa and 105 kDa) can stimulate the downregulation of the PDGFR (and its signaling) via the lysosomal degradation pathway in NIH 3T3 cells (Ignatiuk *et al*., 2006). Now we performed a similar study of the effect of ankyrin105 on EGFR degradation. Before the study was carried out, we tested the endogenous expression of ankyrin3 in COS-1, HEK293T, MCF10A, MDA-MB-231 and AU565 cells, which were the model cell lines chosen for the study.

For this experiment, cells from the five selected cell lines were collected in SDS sample buffer. The whole cell lysates were resolved by SDS-PAGE, transferred to nitrocellulose membranes and probed with antibodies against ankyrin3, EGFR, ErbB2 and GAPDH (Figure 4.1). Then infrared dye-labeled secondary antibodies were used to detect bound antibodies using an Odyssey Infrared Imaging System. For the blots probed with ankyrin3 and GAPDH, 100 μg of protein was loaded. For the blots probed with EGFR and ErbB2, 50 μg of protein was loaded.

Different cell lines expressed different isoforms of ankyrin3 (Figure 4.1). The prestained markers used are only an approximation of the molecular weights. Besides, ankyrin3 isoforms may have some translational modifications that may slightly change their sizes. So we indicated the molecular weights of ankyrin3 isoforms according to estimation in Figure 4.1. In COS-1, HEK293T and AU565 cells, there were relatively low expression of the large isoform of ankyrin3 and high expression of ankyrin120 and ankyrin105. In MCF10A and MDA-MB-231 cells, almost no major isoforms of ankyrin3 were detected. However, MDA-MB-231 cells showed a relatively low level of ankyrin120 and ankyrin105. The immunoblot for GAPDH antibody was shown as a loading control to make sure that similar amounts of total protein were loaded. In conclusion, there was high endogenous ankyrin105 expression in COS-1, HEK293T

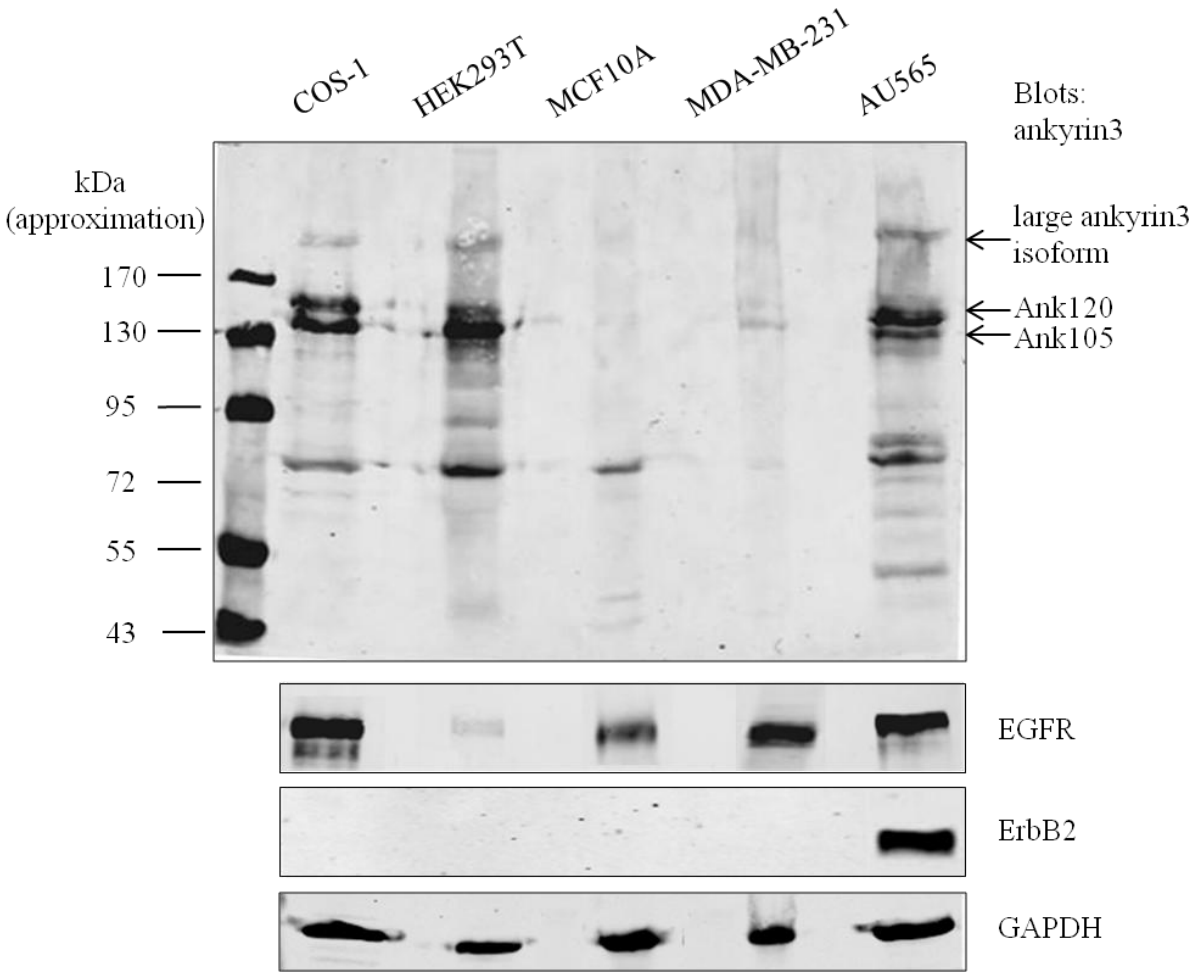

Figure 4.1 Ankyrin3, EGFR and ErbB2 expression in selected cell lines. Cells were collected in SDS sample buffer. The whole cell lysates were resolved by SDS-PAGE, transferred to nitrocellulose membrane and probed with the indicated antibodies. Then infrared dye-labeled secondary antibodies were used and bound antibodies were visualized using an Odyssey Infrared Imaging System. For the blots probed with ankyrin3 and GAPDH, 100 μg of protein was loaded. For the blots probed with EGFR and ErbB2, 50 μg of protein was loaded. Prestained molecular weight markers were shown on the left and their approximate molecular weights were indicated. Representative blots were shown for one of two independent experiments.

and AU565 cells and low endogenous ankyrin105 expression in MDA-MB-231 cells.

As shown in Figure 4.1, these selected cell lines all expressed EGFR, but ErbB2 was only detected in AU565 cells at a relatively high level. In COS-1, MDA-MB-231 and AU565 cells, EGFR levels were relatively high compared to other 2 cell lines. MCF10A cells expressed less EGFR and HEK293T cells had the least EGFR expression among 5 cell lines.

4.2 Overexpression of Ankyrin105 in COS-1 Cells

COS-1 cells are transformed African green monkey kidney cells and are commonly used for transfection to obtain high transient expression of recombinant proteins. COS-1 cells have SV40 genomic insertion which can express large T antigen. This promotes the plasmids containing an SV40 replication origin to replicate by inactivating tumor suppressors and regulating cyclins and cyclin-dependent kinases (Ali and DeCaprio, 2001; Asano *et al*., 1985).

In order to get high expression of ankyrin105 in COS-1 cells, we transfected the cells with pHA_3-Ank105 plasmid (containing an SV40 replication origin) using Lipofectamine reagent. After serum starvation for 16 to 24 hours, COS-1 parental cells and cells transfected with different amounts (3 μg and 6 μg) of HA-Ank105 plasmid DNA were treated with EGF in a time course experiment (0 minute, 30 minutes and 120 minutes). The cell lysates (25 μg of protein/lane) were analyzed by Western blot with EGFR, pTyr, pAkt/Akt, pMAPK/MAPK, HA and GAPDH antibodies.

In parental cells, EGFRs were degraded in response to EGF stimulation (Figure 4.2). The activation of EGFRs can be measured by the phosphotyrosine level using an antibody specific for phosphotyrosine residues. The pTyr blot showed the activation of EGFRs, which was slightly downregulated upon EGF stimulation in the time course experiment up to 120 minutes. The immunoblot for HA antibody was used to confirm the expression of HA-Ank105 for different time points. HA-Ank105 expression did not have an impact on basal and activated EGFR levels. In parental and ankyrin105-expressing cells, it was surprising that pAkt levels were so low at 30 minutes and so high at 120 minutes, which might suggest the upregulation of PI3K-Akt pathway. pMAPK results were fairly typical that MAPK was activated in response to

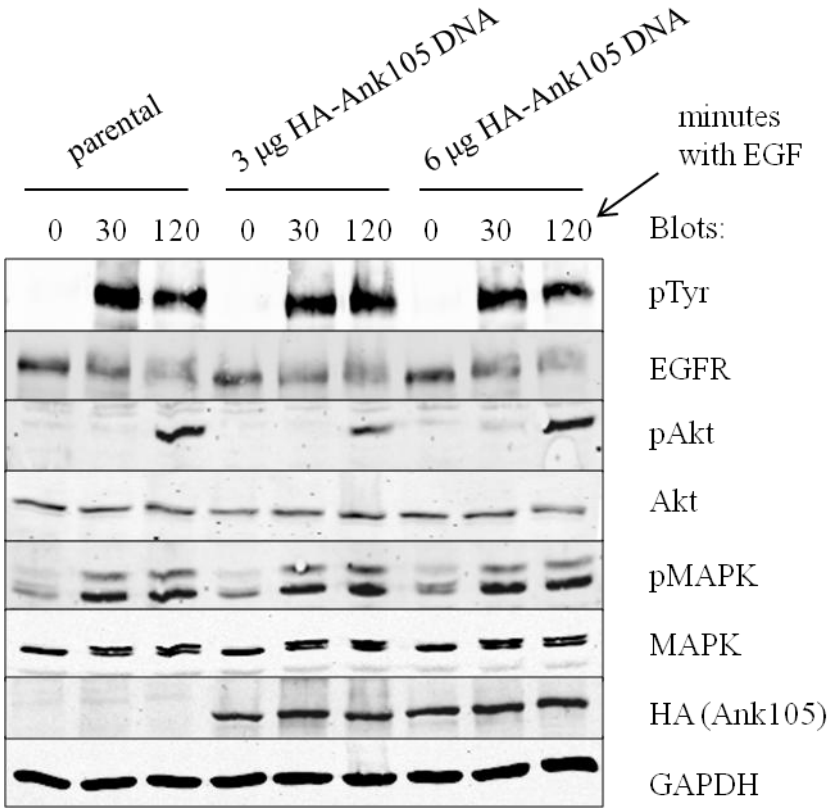

Figure 4.2 Effect of ankyrin105 overexpression on EGFR and signaling pathways in COS-1 cells. Time course of EGF stimulation (0 min, 30 min and 120 min) in COS-1 parental cells and cells transfected with different amounts of HA-Ank105 plasmid DNA was carried out. The cell lysates (25 µg of protein/lane) were analyzed by Western blot with EGFR, pTyr, pAkt/Akt, pMAPK/MAPK, HA and GAPDH antibodies. Representative blots were shown for one of three independent experiments.

EGF stimulation and showed a sustained activation. The immunoblot for GAPDH antibody was shown as a loading control. In summary, overexpression of ankyrin105 did not enhance EGFR degradation or downregulation of EGFR signaling pathways in COS-1 cells.

4.3 Overexpression of Ankyrin105 in HEK293T Cells

4.3.1 Optimization of Calcium Phosphate Transfection Efficiency

HEK293T cells are transformed human embryonic kidney cells and are easily transfected by various methods, such as calcium phosphate. Like COS-1 cells, HEK293T cells also have SV40 genomic insertion, so they could take the advantage of large T antigen to help the plasmids containing SV40 replication origin to multiply.

Calcium phosphate transfection of HEK293T cells can generate relatively high transient expression levels, but the amount of plasmid DNA used for transfection needs to be optimized. In this experiment, HEK293T cells were transfected with different amounts (3 μg, 5 μg, 6.25 μg, 7 μg and 9 μg) of pEGFP-C1 plasmid (containing SV40 replication origin) in a 6-well plate using calcium phosphate transfection methods. After 16 hours incubation, the media containing pEGFP-C1 plasmid was removed, and then the cells were washed and fed. To monitor GFP expression, the cells were photographed 2 days post-transfection under fluorescence microscopy. We compared the cells using fluorescent (Figure 4.3 left panel) and bright field modes (Figure 4.3 right panel).

For different transfection conditions (i.e. the amounts of plasmid DNA), the numbers of total cells were similar (Figure 4.3 right panel). The number of cells that expressed GFP representing transfection efficiency increased with the increasing amounts of GFP DNA up to 5 μg (Figure 4.3 left panel). When the DNA amount was greater than 5 μg, the number of GFP-expressing cells decreased, which suggested that transfection efficiency gradually decreased with the increasing DNA amount. It seemed that large amounts of DNA were toxic to the cells. The GFP expression was optimal using 5 μg plasmid DNA, which was used for further experiments.

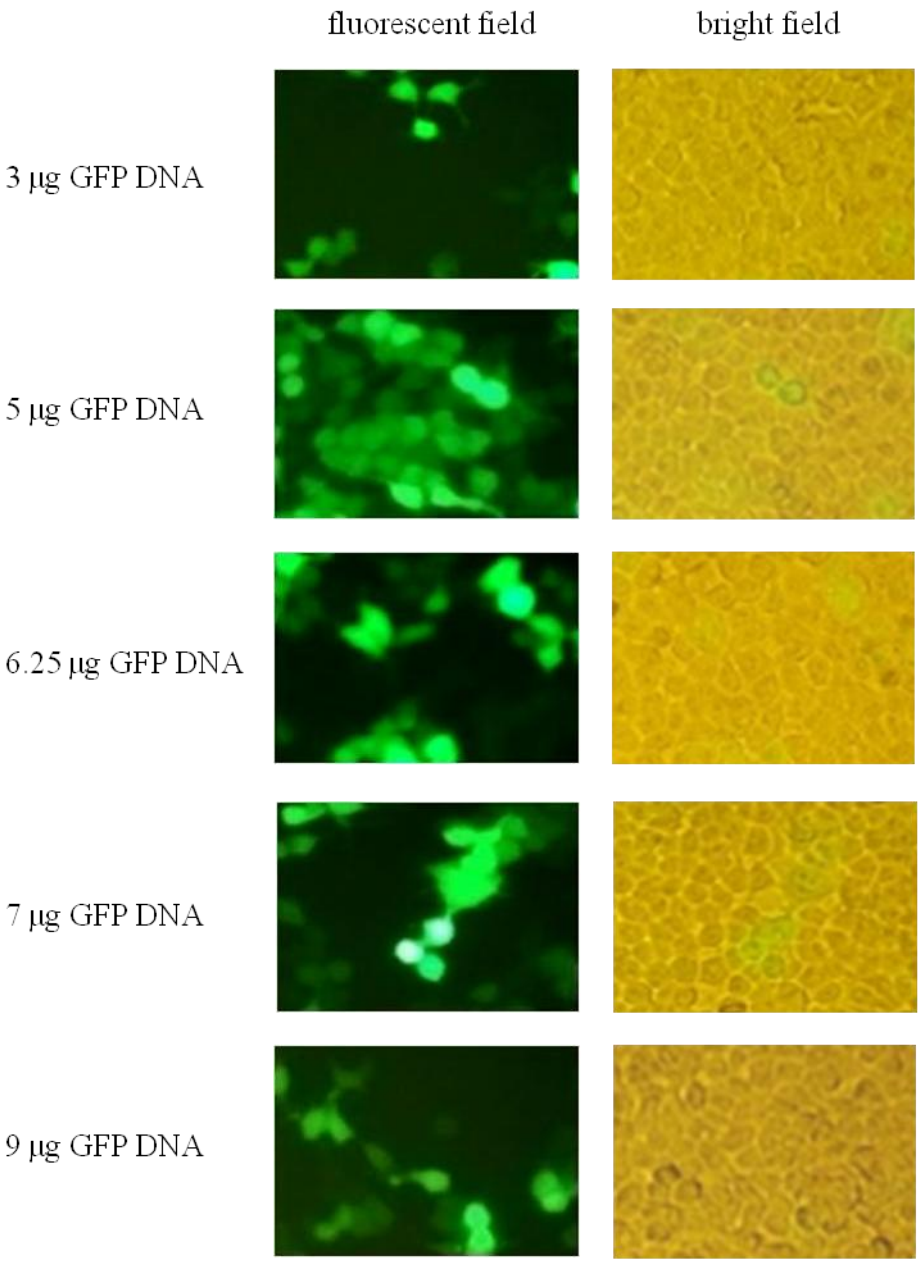

Figure 4.3 Optimization of calcium phosphate transfection efficiency in HEK293T cells. HEK293T cells were transfected with different amounts of GFP DNA in a 6-well plate using calcium phosphate. Cells were photographed 2 days post-transfection to monitor GFP expression under fluorescence microscopy using fluorescent (left panel) and bright field modes (right panel). Representative photos were shown for one of two independent experiments.

4.3.2 Effect of Ankyrin105 Overexpression on Receptor Tyrosine Kinases and Signaling Pathways in HEK293T Cells

To address ankyrin105 function in the degradation of EGFR in HEK293T cells, the cells were transfected using optimized amount (30 μg for 10 cm plate) of pHA_3 and pHA_3-Ank105 plasmids (both containing an SV40 replication origin) by calcium phosphate. The cells transfected with pHA_3 plasmid were used as controls to make sure that calcium phosphate transfection process and HA expression wound not impact the properties of the cells. After the transfection media was removed and the cells were washed and fed for 8 hours, the cells were serum starved for 16 hours. Then HEK293T parental cells and cells transfected with HA and HA-Ank105 were treated in a time course EGF stimulation experiment. The cell lysates were analyzed by Western blot with the indicated antibodies. For the blots probed with EGFR and pTyr, 200 μg of protein was loaded. For other blots, 25 μg of protein was loaded. This is because HEK293T cells express a relatively low level of EGFR compared to other selected cell lines (Figure 4.1), which makes it difficult to detect the changes in EGFR levels during the stimulation.

In parental cells, EGFRs were rapidly degraded in response to EGF stimulation (Figure 4.4). After 30 minutes, there were almost no EGFRs left. The corresponding phosphotyrosine blots suggested the downregulation of activated EGFRs and the weak bands were probably due to the low EGFR expression in this cell line. The immunoblots for HA antibody were used to confirm that the transfection efficiency was similar among the different time point samples. In ankyrin105-expressing cells, both EGFR and activated EGFR levels decreased in the time course treatment. However, there was no significant difference in EGFR degradation when comparing to parental cells and HA-expressing cells. In each of the three cell lines, PI3K-Akt pathway showed a sustained activation, while Ras-MAPK pathway showed a decreased activation in signaling as compared to earlier time points in response to EGF stimulation. Akt activation was upregulated in both HA and HA-Ank105 transfected cells compared to parental

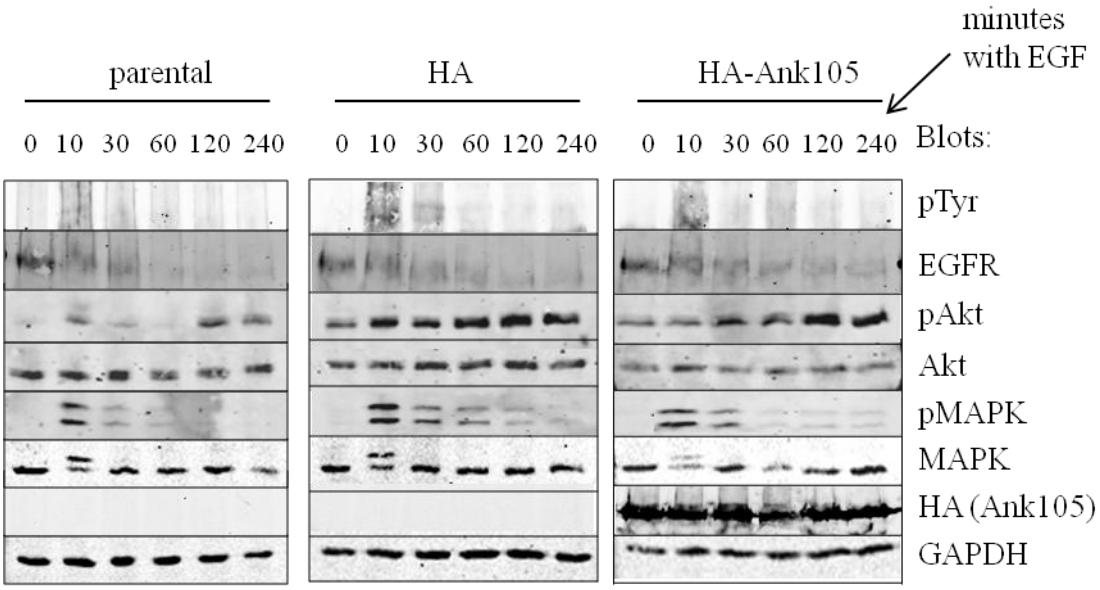

Figure 4.4 Effect of ankyrin105 overexpression on EGFR and signaling pathways in HEK293T cells. Time course of EGF stimulation (0 min, 10 min, 30 min, 60 min, 120 min and 240 min) in HEK293T parental cells and cells transfected with HA and HA-Ank105 was carried out. The cell lysates were analyzed by Western blot with EGFR, pTyr, pAkt/Akt, pMAPK/MAPK, HA and GAPDH antibodies. For the blots probed with EGFR and pTyr, 200 μg of protein was loaded. For other blots, 25 μg of protein was loaded. Representative blots were shown for one of three independent experiments.

cells. The immunoblots for GAPDH antibody were shown as a loading control. In conclusion, overexpression of ankyrin105 did not enhance EGFR degradation or downregulation of EGFR signaling pathways in HEK293T cells.

4.4 Overexpression of Ankyrin105 in MCF10A Cells

4.4.1 Optimization of Adenovirus Transduction Efficiency in MCF10A Cells

MCF10A cells are immortalized human breast cells, but are not tumorigenic or transformed (Debnath *et al*., 2003; Imbalzano *et al*., 2009). In order to get high expression of ankyrin105, an adenovirus vector was used because it could deliver the recombinant viral DNA into the nucleus of host cell and replicate in both dividing and non-dividing cells (Kawabata *et al*., 2006; Tashiro *et al*., 2010).

To decide how much adenovirus was required to obtain high HA-Ank105 expression, different MOIs of Ad-GFP were tested first. MCF10A cells (7.5×10^4) were plated in 6-well plate the day before transduction. The cells were about 50% confluent by the next day. The cells were incubated in adenoviral media containing Ad-GFP at designed MOIs (50, 100 and 200) for 1 hour. A second transduction was performed 2 days after the first transduction. The cells expressing GFP and not expressing GFP were counted under fluorescence and bright field microscopy 5 days after the first transduction to determine transduction efficiency.

The transduction efficiency was shown as a percentage and it increased with increasing MOI (Table 4.1). At an MOI 200, more than 85% of cells were transduced. However, transducing the cells twice did not significantly improve the transduction efficiency. Therefore, an MOI 200 and single transduction were selected for further experiments to balance the transduction efficiency with the amount of adenovirus used.

To determine the expression of transduced viral DNA, MCF10A cells were transduced with Ad-GFP+HA-Ank105 at an MOI 200. This adenovirus vector coexpresses GFP and HA-Ank105. The cells were photographed under fluorescence microscope every day post-transduction up to 5 days to monitor GFP expression (Figure 4.5 A). The cell lysates from

Table 4.1 Optimization of adenovirus transduction efficiency in MCF10A cells. MCF10A cells were transduced once or twice (2 days after the first transduction) by a control adenovirus expressing GFP at different MOIs. Cells expressing GFP and not expressing GFP were counted 5 days after the first transduction. Similar results were obtained in two independent experiments.

MOI (transduction once)	**Efficiency**	**MOI (transduction twice)**	**Efficiency**
50	68%	50/50	72%
100	77%	100/100	82%
200	85%	200/200	88%

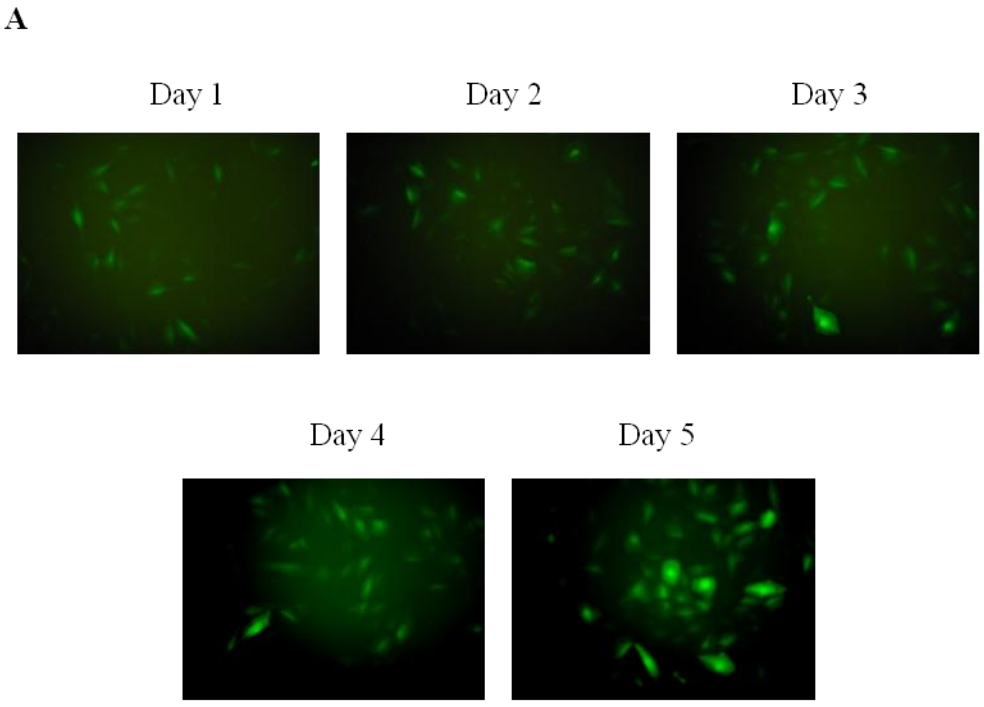

Figure 4.5 Optimization of adenovirus transduction efficiency in MCF10A cells. MCF10A cells were transduced by the adenovirus coexpressing GFP and HA-Ank105 at an MOI 200. A, The cells were photographed under fluorescence microscopy each day after transduction to monitor GFP expression. B, The cell lysates (25 μg of protein/lane) were analyzed by Western blot with GFP, HA and GAPDH antibodies. Representative results were shown for one of two independent experiments.

each day post-transduction were analyzed by Western blot with GFP, HA and GAPDH antibodies (Figure 4.5 B). The control sample was MCF10A whole cell lysate.

Both the number of cells expressing GFP and the levels of GFP expression increased with days of post-transduction up to 5 days (Figure 4.5 A). The Western blot results further confirmed increasing levels of GFP and HA-Ank105 expression after transduction (Figure 4.5 B). The cells would be too confluent 5 days post-transduction. Therefore, further experiments were carried out 4 or 5 days post-transduction to get high protein expression and not to lose the cell properties.

4.4.2 Effect of Ankyrin105 Overexpression on Receptor Tyrosine Kinases and Signaling Pathways in MCF10A Cells

To characterize ankyrin105 function in the degradation of EGFR in MCF10A cells, GFP and GFP+HA-Ank105 were transduced into the cells by adenovirus using the optimal conditions determined in previous experiments. The cells transduced with Ad-GFP were used as a control to make sure that the adenovirus transduction process and GFP expression wound not impact the properties of the cells. The cells were serum starved for 16 to 24 hours on the fourth day post-transduction. Then MCF10A parental cells, Ad-GFP transduced cells and Ad-GFP+HA-Ank105 transduced cells were treated with EGF in a time course experiment. The cell lysates were analyzed by Western blot with the indicated antibodies.

As shown in Figure 4.6, EGFRs were rapidly degraded in response to EGF stimulation in parental cells. After 60 minutes, EGFRs were barely detected. GFP blots confirmed the transduction efficiency and HA blots confirmed the expression of HA-Ank105 was similar among the different time points. In both transduced cells, the rate of EGFR degradation was slower than that in parental cells. The corresponding phosphotyrosine blots indicated the downregulation of activated EGFRs. In all three cell lines, both PI3K-Akt pathway and Ras-MAPK pathway showed a decreased activation in signaling as compared to earlier time points upon EGF stimulation, which indicated that the downregulation of downstream signaling

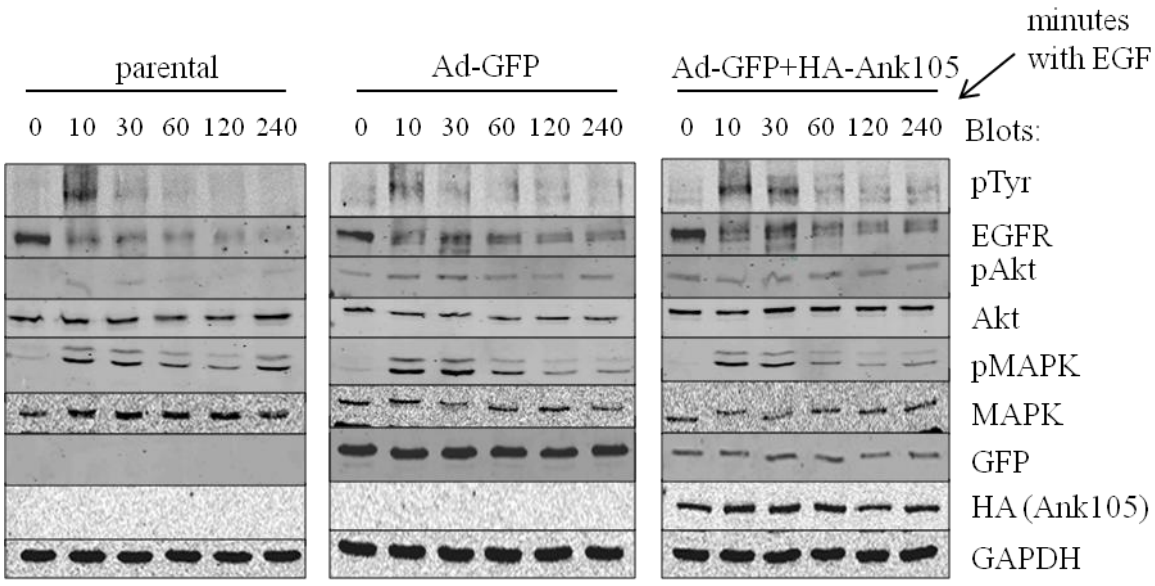

Figure 4.6 Effect of ankyrin105 overexpression on EGFR and signaling pathways in MCF10A cells. Time course of EGF stimulation (0 min, 10 min, 30 min, 60 min, 120 min and 240 min) in MCF10A parental cells, cells transduced with adenovirus GFP control and with adenovirus GFP+HA-Ank105 was carried out. The cell lysates (25 μg of protein/lane) were analyzed by Western blot with EGFR, pTyr, pAkt/Akt, pMAPK/MAPK, GFP, HA and GAPDH antibodies. Representative blots were shown for one of three independent experiments.

pathways were not affected by ankyrin105. The immunoblots for GAPDH antibody were shown as a loading control. These results suggested that overexpression of ankyrin105 did not enhance EGFR degradation or downregulation of EGFR signaling pathways in MCF10A cells.

4.5 Overexpression of Ankyrin105 in MDA-MB-231 Cells

4.5.1 Optimization of Adenovirus Transduction Efficiency in MDA-MB-231 Cells

MDA-MB-231 cells are highly invasive and metastatic human breast cancer cells (Laezza *et al*., 2012). In order to get high expression of ankyrin105, an adenovirus vector was used to deliver the desired gene.

To decide how much adenovirus was required to obtain high HA-Ank105 expression, different MOIs were first tested. MDA-MB-231 cells (3×10^5) were plated in a 6-well plate the day before transduction. The following day 50% confluent cells were incubated in adenoviral media containing Ad-GFP at designed MOIs (100, 200 and 500) for 1 hour. Then the cells were photographed using fluorescence microscopy with both fluorescent and bright field every day post-transduction up to 4 days to determine transduction efficiency and GFP expression (Figure 4.7). Both the number of cells expressing GFP and GFP expression increased with the increasing MOIs and days of post-transduction up to 4 days. On the fourth day, transduction efficiency and GFP expression between MOI 200 and MOI 500 were almost the same and majority of the cells were transduced at MOI 200. Therefore, a higher MOI was not necessary and an MOI 200 was chosen for further experiments.

4.5.2 Effect of Ankyrin105 Overexpression on Receptor Tyrosine Kinases and Signaling Pathways in MDA-MB-231 Cells

To determine ankyrin105 function in the degradation of EGFR in MDA-MB-231 cells, GFP and GFP+HA-Ank105 were transduced into the cells by adenovirus at an MOI 200. The cells were serum starved for 16 to 24 hours on the fourth day post-transduction. Then MDA-MB-231 parental cells, Ad-GFP transduced cells and Ad-GFP+HA-Ank105 transduced

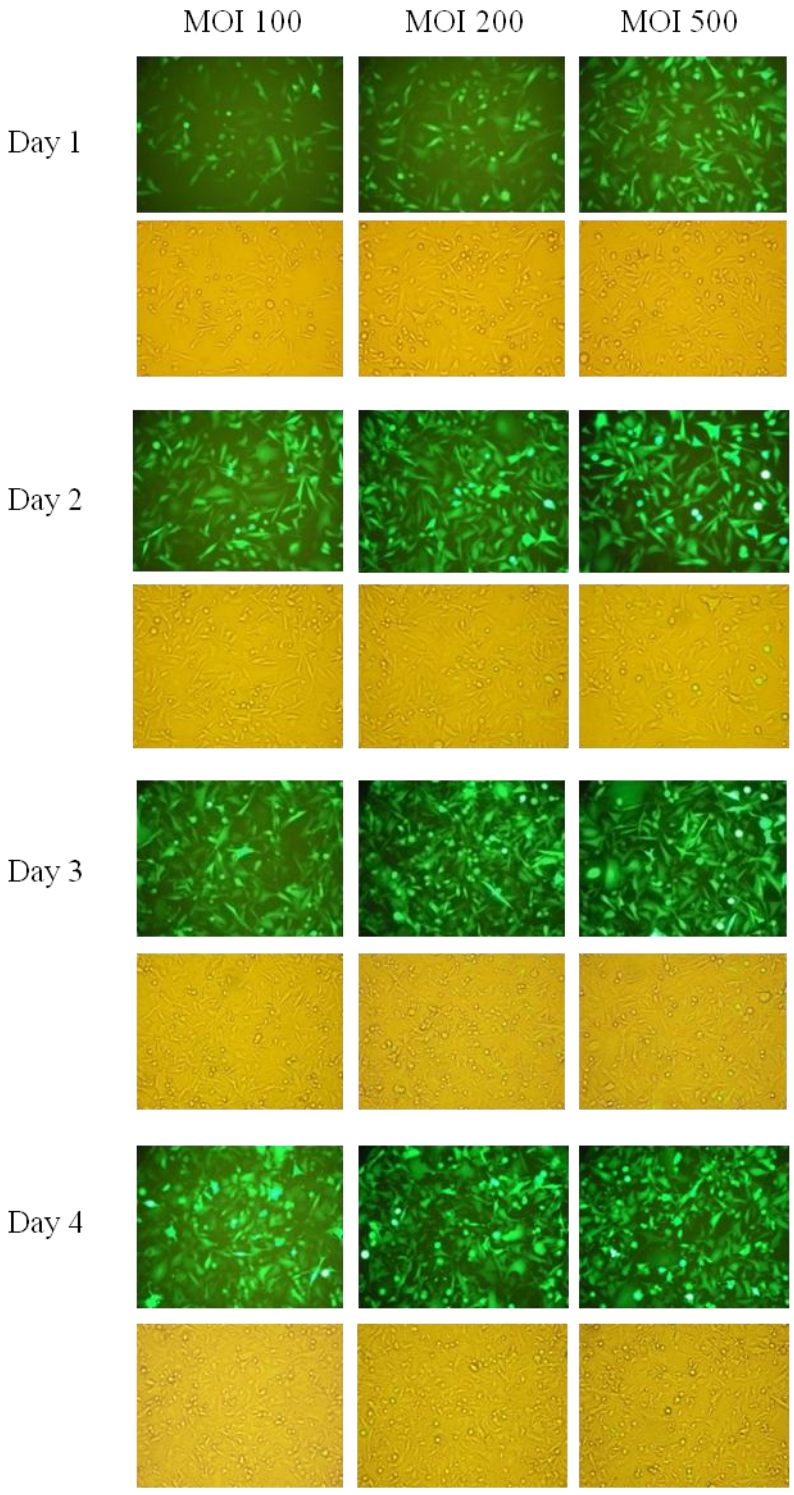

Figure 4.7 Optimization of adenovirus transduction efficiency in MDA-MB-231 cells. MDA-MB-231 cells were transduced by a control adenovirus (Ad-GFP) at different MOIs. Cells were photographed every day post-transduction up to 4 days to monitor GFP expression. Representative photos were shown for one of two independent experiments.

cells were treated with EGF for different times. The cell lysates were analyzed by Western blot with the indicated antibodies.

Surprisingly, EGFRs were not degraded in response to EGF stimulation in parental cells (Figure 4.8). However, the corresponding phosphotyrosine blots showed the downregulation of activated EGFRs, which was not correlated to EGFR level. In both transduced cells, EGFR level did not change, which was almost the same as that in parental cells. The activated EGFRs downregulated very fast and were hardly detected even after 10 minutes of stimulation. In all three cell lines, PI3K-Akt pathway showed prolonged activation, while Ras-MAPK pathway showed a decreased activation in signaling as compared to earlier time point during EGF stimulation. But the downregulation of activated MAPK was facilitated in both transduced cells compared to parental cells. The immunoblots for GFP, HA and GAPDH antibodies were shown as control. Overexpression of ankyrin105 did not enhance EGFR degradation or downregulation of EGFR signaling pathways in MDA-MB-231 cells.

4.6 Overexpression of Ankyrin105 in AU565 Cells

4.6.1 Optimization of Adenovirus Transduction Efficiency in AU565 Cells

AU565 cells are human breast cancer cells and express high levels of both EGFR and ErbB2 (Figure 4.1) (Huang *et al*., 2010; Neve *et al*., 2006). Similar to other human breast cell lines, adenovirus vector was transduced into cells to get high expression of ankyrin105.

To optimize how much adenovirus was required for high HA-Ank105 expression, different MOIs were tested. AU565 cells (7.5×10^4) were plated in a 6-well plate the day before transduction. The following day 50% confluent cells were incubated in adenoviral media containing Ad-GFP control at designed MOIs for 1 hour. Then the cells were photographed using fluorescence microscopy every day post-transduction up to 4 days to determine the optimal conditions (Figure 4.9). Both the number of cells expressing GFP and GFP expression increased with the increasing MOIs and days of post-transduction up to 4 days. There were not significant differences in transduction efficiency and GFP expression between MOI 200 and

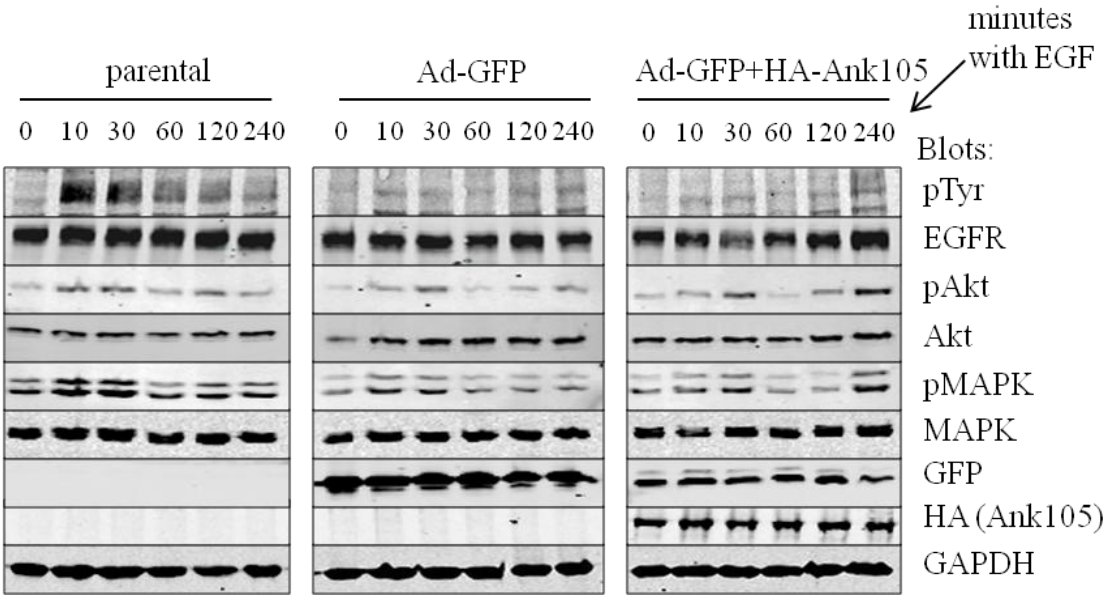

Figure 4.8 Effect of ankyrin105 overexpression on EGFR and signaling pathways in MDA-MB-231 cells. Time course of EGF stimulation (0 min, 10 min, 30 min, 60 min, 120 min and 240 min) in MDA-MB-231 parental cells, cells transduced with adenovirus GFP control and with adenovirus GFP+HA-Ank105 was carried out. The cell lysates (25 μg of protein/lane) were analyzed by Western blot with EGFR, pTyr, pAkt/Akt, pMAPK/MAPK, GFP, HA and GAPDH antibodies. Representative blots were shown for one of three independent experiments.

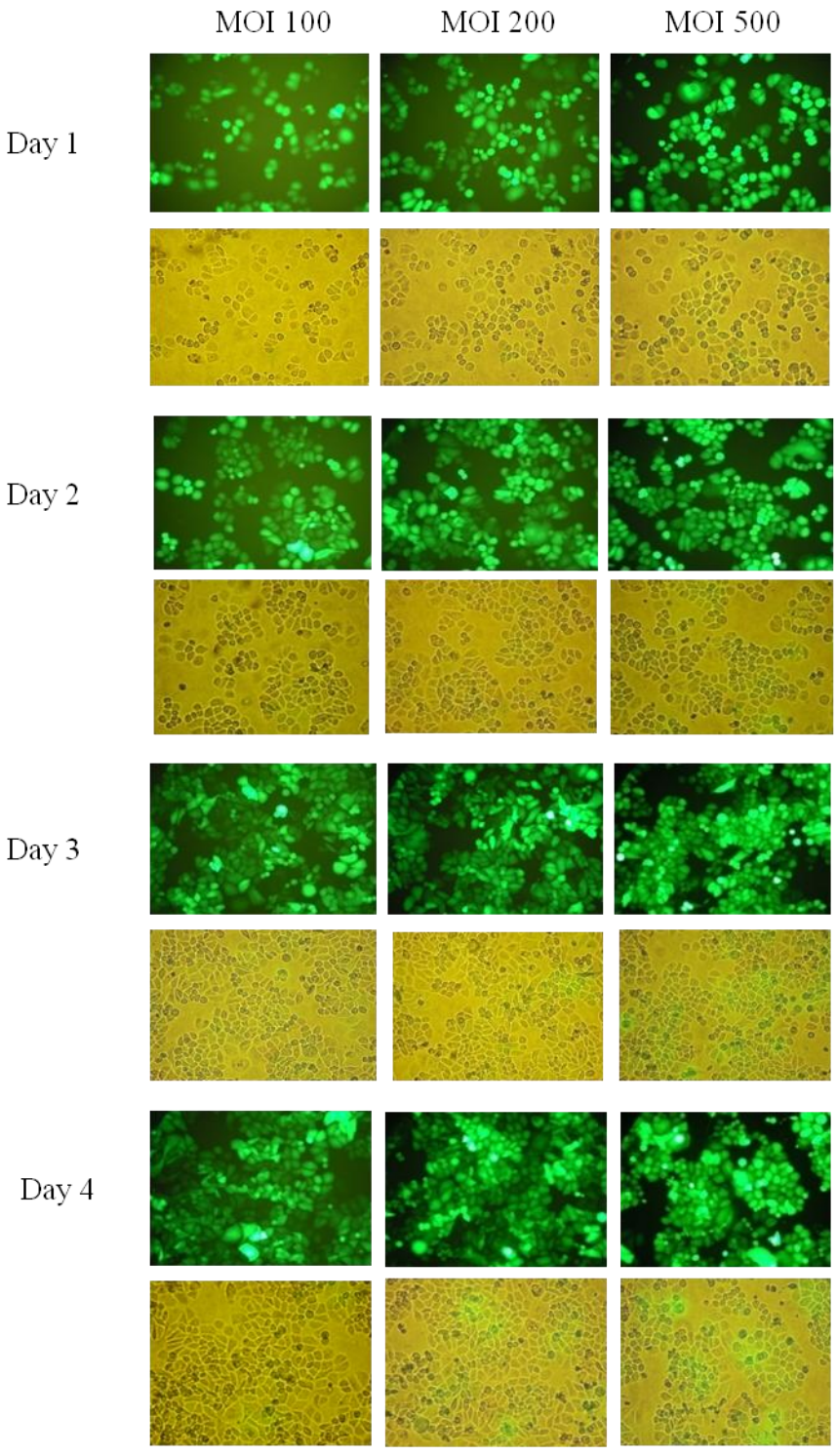

Figure 4.9 Optimization of adenovirus transduction efficiency in AU565 cells. AU565 cells were transduced by a control adenovirus (Ad-GFP) at different MOIs. Cells were photographed every day post-transduction up to 4 days to monitor GFP expression. Representative photos were shown for one of two independent experiments.

MOI 500. So an MOI 200 was chosen for the further study as previous experiments.

4.6.2 Effect of Ankyrin105 Overexpression on Receptor Tyrosine Kinases and Signaling Pathways in AU565 Cells

To identify ankyrin105 function in the degradation of EGFR in AU565 cells, GFP and GFP+HA-Ank105 were transduced into the cells by adenovirus at an MOI 200. The cells were serum starved for 16 to 24 hours on the fourth day post-transduction. Then AU565 parental cells, Ad-GFP transduced cells and Ad-GFP+HA-Ank105 transduced cells were stimulated with EGF for indicated times. The cell lysates were analyzed by Western blot with the indicated antibodies.

In parental cells, both EGFRs and ErbB2 were not degraded in response to EGF stimulation (Figure 4.10). There was a basal level of activated EGFRs prior to EGF stimulation and activated EGFRs were slowly downregulated. Cells transduced with Ad-GFP+HA-Ank105 did not show significant differences for EGFR, activated EGFR and ErbB2 compared to parental cells and control adenovirus transduced cells. The downregulation of EGFR downstream signaling (pAkt/Akt, pMAPK/MAPK) was promoted in both Ad-GFP and Ad-GFP+HA-Ank105 transduced cells compared to parental cells. GFP, HA and GAPDH blots were shown as control. In summary, overexpression of ankyrin105 did not enhance EGFR degradation or downregulation of EGFR signaling pathways in AU565 cells.

4.6.3 Effect of Geldanamycin Treatment on Receptor Tyrosine Kinases

ErbB2 can heterodimerize with other members of the EGFR subfamily without growth factors (Burgess *et al*., 2003). It is shown to be internalization resistant (Hommelgaard *et al*., 2004). So overexpression of ErbB2 in AU565 cells may block internalization, endocytosis and degradation of receptors. In order to downregulate ErbB2 and reduce ErbB2-mediated blocking of EGFR degradation, an Hsp90 inhibitor geldanamycin was used.

To investigate to what extent geldanamycin can induce internalization of ErbB2, AU565

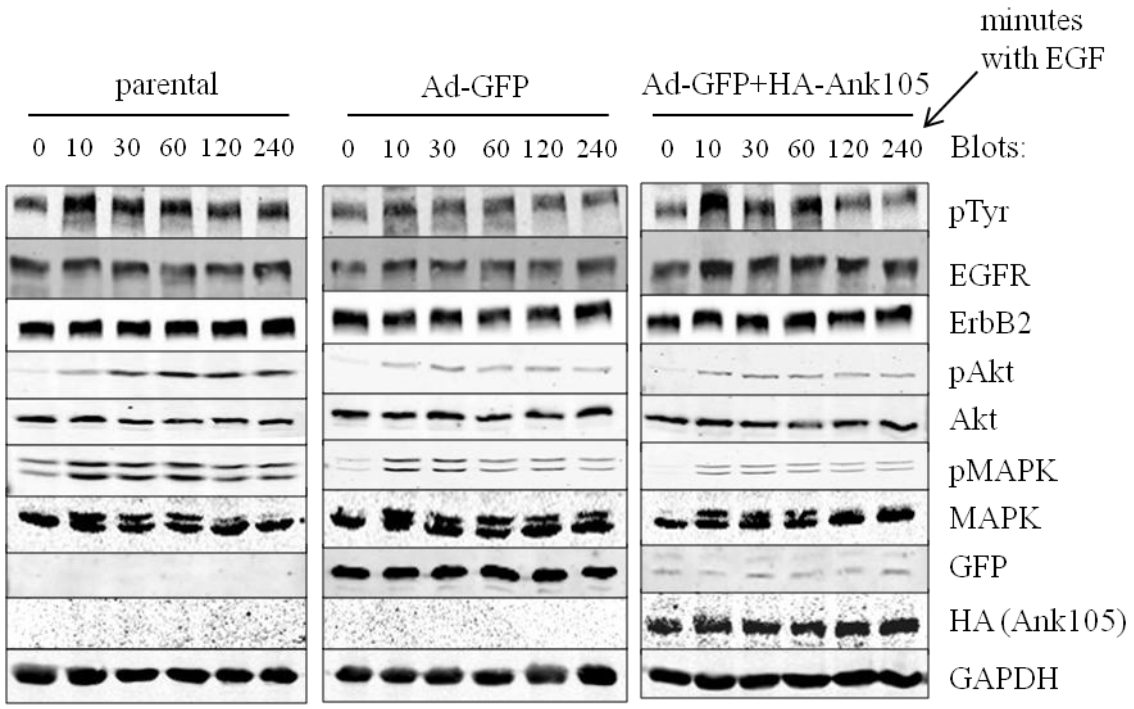

Figure 4.10 Effect of ankyrin105 overexpression on ErbB2, EGFR and signaling pathways in AU565 cells. Time course of EGF stimulation (0 min, 10 min, 30 min, 60 min, 120 min and 240 min) in AU565 parental cells, cells transduced with adenovirus GFP control and with adenovirus GFP+HA-Ank105 was carried out. The cell lysates (25 μg of protein/lane) were analyzed by Western blot with EGFR, ErbB2, pTyr, pAkt/Akt, pMAPK/MAPK, GFP, HA and GAPDH antibodies. Representative blots were shown for one of six independent experiments.

cells were treated with DMSO (control; Geldanamycin is made up in DMSO), GA (1 μmol/L), EGF or GA+EGF for different times (0 h, 2 h, 4 h, 6 h, 12 h and 24 h) at 37ºC. After fixation, the cells were incubated with an antibody specific to the extracellular domain of ErbB2. Then a PE-conjugated secondary antibody was added. Flow cytometry analysis was used to detect cell surface ErbB2 (Figure 4.11).

When incubated with DMSO or EGF for different times, the peak representing surface ErbB2 did not shift, which suggested that ErbB2 was not internalized (Figure 4.11 A). When treated with GA or GA+EGF for more than 4 hours, the shift to the left in the peak showed that geldanamycin induced a decrease in surface ErbB2. In Figure 4.11 B, the same flow cytometry data were analyzed by comparing different treatments at the same time point. It was much clearer that ErbB2 was internalized by geldanamycin treatment. However, there was not much difference between cells treated with GA alone and GA+EGF together. EGF did not promote geldanamycin-induced ErbB2 internalization. The flow cytometry data of cells and cells treated with only secondary antibody were shown as negative controls. The flow cytometry results confirmed that ErbB2 could be internalized upon geldanamycin treatment.

To investigate to what extent geldanamycin could induce degradation of ErbB2, AU565 cells were incubated with DMSO, GA, EGF or GA+EGF for various times. Cell lysates were analyzed by Western blot with EGFR, ErbB2 and GAPDH antibodies to detect the degradation of ErbB2 and EGFR. The immunoblot results showed that ErbB2 was degraded in response to geldanamycin treatment and EGFRs were also degraded in parallel with the decreased ErbB2 levels (Figure 4.12). After 12 hours, most ErbB2 and EGFRs were degraded. Similar to flow cytometry results, DMSO or EGF treatment did not change the receptors level and GA+EGF treatment did not further facilitate the degradation of receptors. Therefore, geldanamycin induced degradation of ErbB2 and EGFR in AU565 cells.

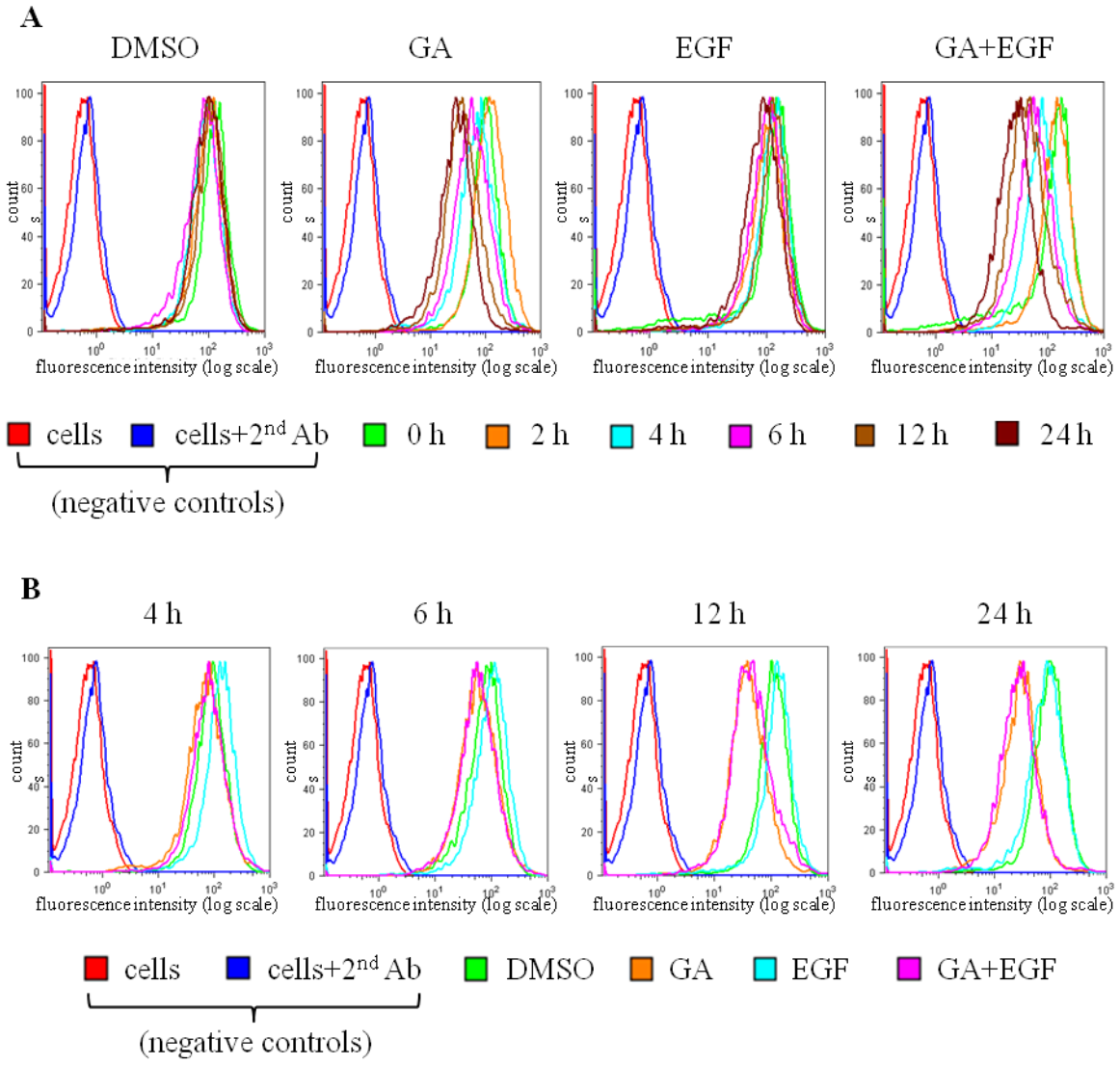

Figure 4.11 Effect of geldanamycin on surface ErbB2 in AU565 cells. AU565 cells were incubated with DMSO, GA (1 μmol/L), EGF or GA+EGF for various times (0 h, 2 h, 4 h, 6 h, 12 h and 24 h) at 37ºC. After fixation, the cells were incubated with an antibody specific to the extracellular domain of ErbB2. Then a PE-conjugated secondary antibody was added. Flow cytometry analysis was used to detect the amount of ErbB2 remaining at the plasma membrane. **A**, Data were analyzed by comparing different time points for the same treatment. **B**, Data were analyzed by comparing different treatments at the same time point. Representative results were shown for one of two independent experiments.

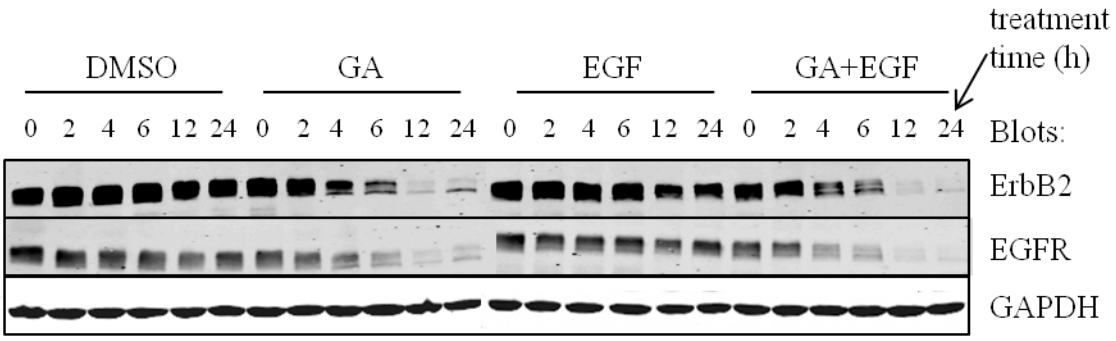

Figure 4.12 Effect of geldanamycin on ErbB2 and EGFR in AU565 cells. AU565 cells were incubated with DMSO, GA (1 μmol/L), EGF or GA+EGF for various times at 37ºC. The cell lysates (25 μg of protein/lane) were analyzed by Western blot with EGFR, ErbB2 and GAPDH antibodies to detect the degradation of ErbB2 and EGFR. Representative blots were shown for one of two independent experiments.

4.6.4 Effect of Geldanamycin Treatment on Receptor Tyrosine Kinases and Signaling Pathways in Ankyrin105 Overexpressing Cells

Since geldanamycin induced degradation EGFR in AU565 cells, ankyrin105 was introduced into the cells before geldanamycin treatment to test whether overexpression of ankyrin105 could further downregulate EGFR in combination with geldanamycin. To perform the experiment, AU565 parental cells, cells transduced with adenovirus GFP control and with adenovirus GFP+HA-Ank105 were incubated with DMSO, GA, EGF or GA+EGF for various times. Then the cell lysates were analyzed by Western blot with the indicated antibodies.

As shown in Figure 4.13, ankyrin105-expressing cells did not show an increase in EGFR degradation or activated EGFR downregulation compared to parental and control transduced cells. In addition, the downstream signaling pathways (pAkt/Akt, pMAPK/MAPK) were not impacted by ankyrin105 (Figure 4.14). These data indicated that overexpression of ankyrin105 did not enhance EGFR degradation or downregulation of EGFR signaling pathways in AU565 cells treated with geldanamycin which already downregulated ErbB2 and EGFR.

4.6.5 Effect of Herceptin Treatment on Receptor Tyrosine Kinases

A second method to downregulate ErbB2 was herceptin treatment. Herceptin is an anti-ErbB2 antibody, which binds to the extracellular domain of ErbB2. It can affect ErbB2 structure and dimerization with other EGFR family members, and may facilitate internalization and degradation of the receptors (Singer *et al*., 2012; Tsang and Finn, 2012).

AU565 cells were incubated with herceptin (10 μg/mL) and two different doses of EGF for various times at 37ºC. It has been demonstrated that receptors were endocytosed through a clathrin-dependent pathway when treated with low concentrations of EGF and the majority of EGFRs were internalized through a clathrin-independent pathway upon high concentrations of EGF stimulation (Sigismund *et al*., 2005). Flow cytometry analysis was used to detect the downregulation of ErbB2 from the plasma membrane. After treatment with herceptin, EGF and

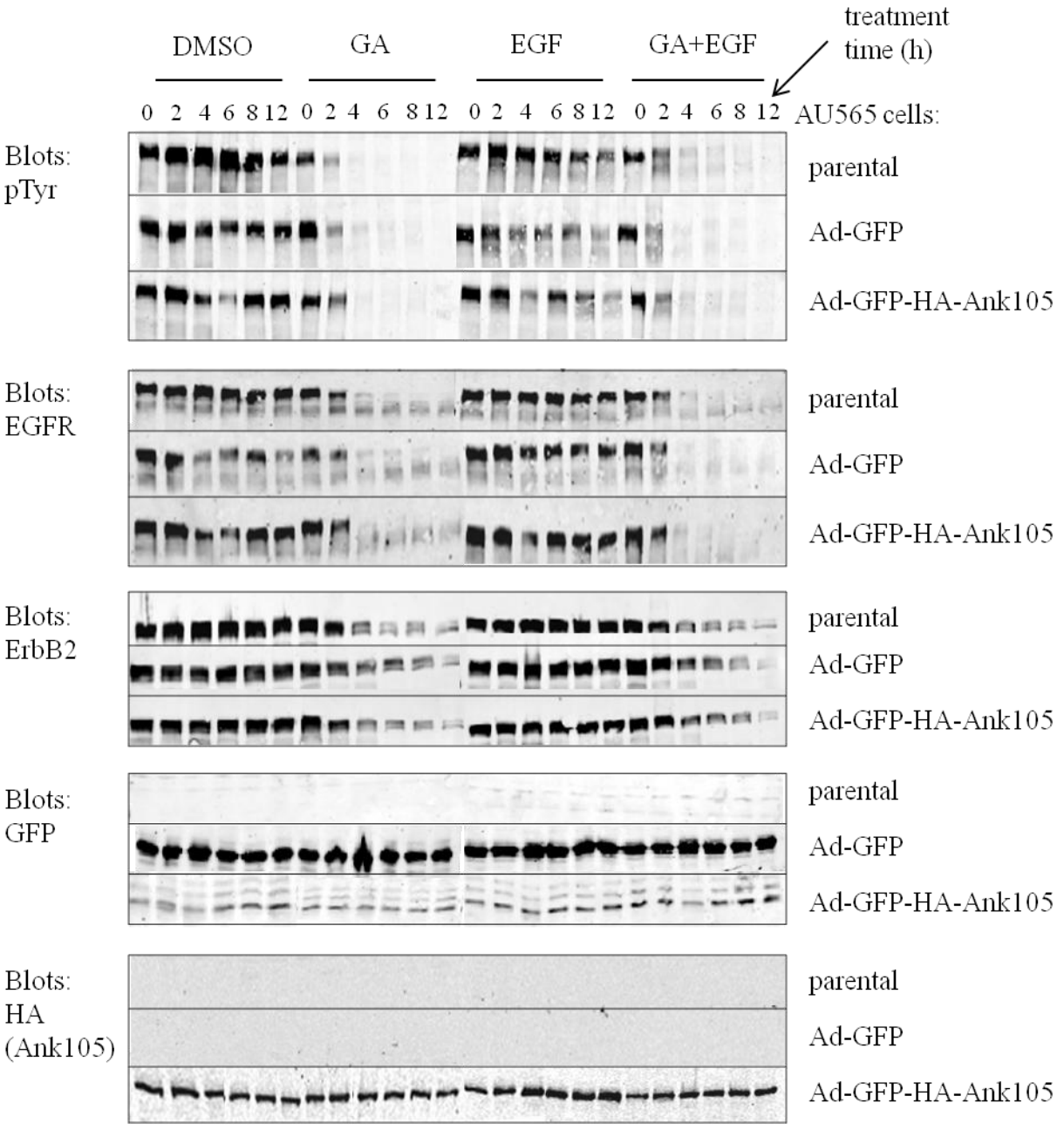

Figure 4.13 Effect of ankyrin105 overexpression on ErbB2 and EGFR in AU565 cells treated with geldanamycin. AU565 parental cells, cells transduced with adenovirus GFP control and with adenovirus GFP+HA-Ank105 were incubated with DMSO, GA (1 μmol/L), EGF or GA+EGF for various times at 37ºC. The cell lysates (25 μg of protein/lane) were analyzed by Western blot with EGFR, ErbB2, pTyr, GFP and HA antibodies. Representative blots were shown for one of three independent experiments.

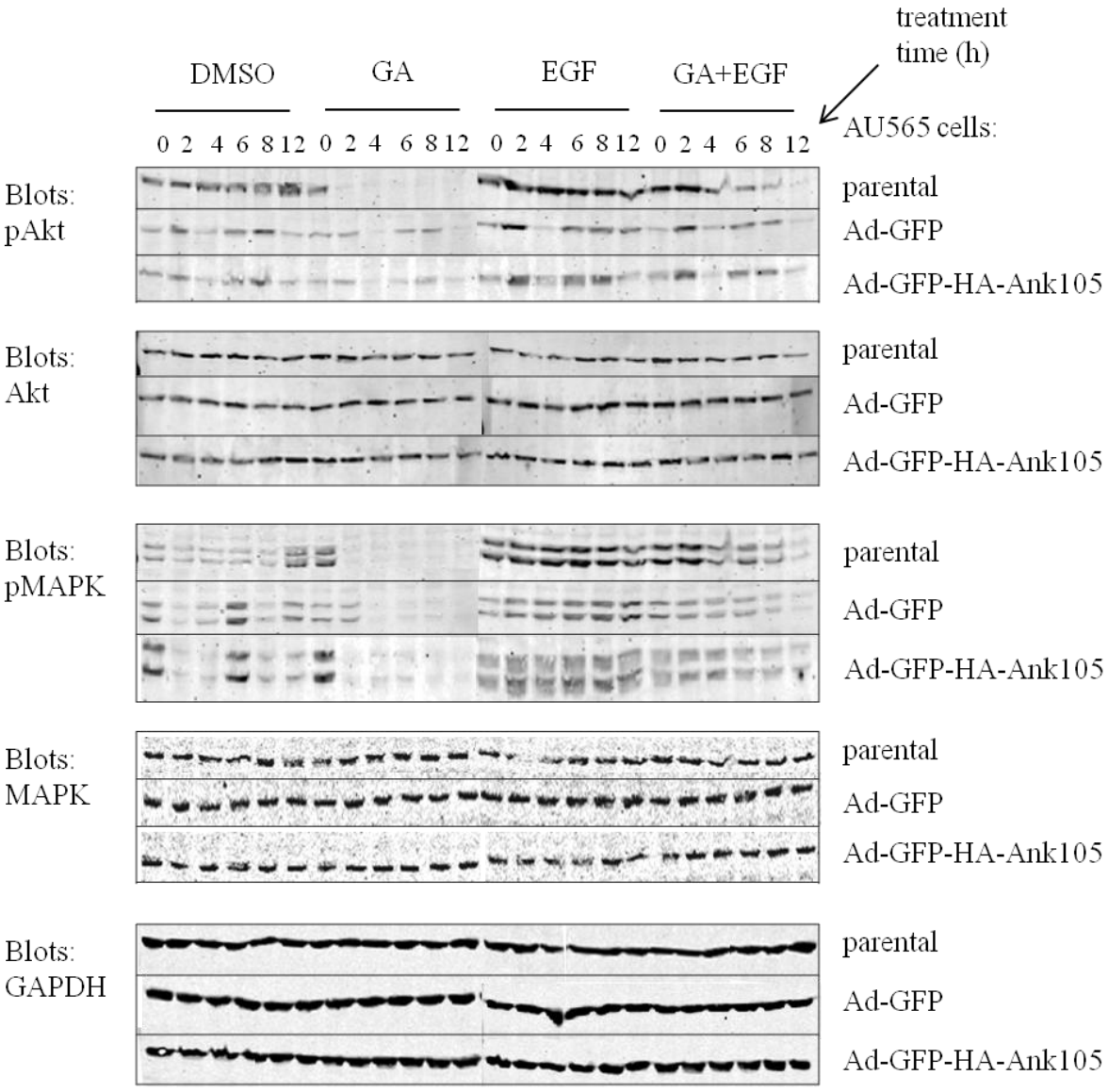

Figure 4.14 Effect of ankyrin105 overexpression on signaling pathways in AU565 cells treated with geldanamycin. AU565 parental cells, cells transduced with adenovirus GFP control and with adenovirus GFP+HA-Ank105 were incubated with DMSO, GA (1 μmol/L), EGF or GA+EGF for various times at 37ºC. The cell lysates (25 μg of protein/lane) were analyzed by Western blot with pAkt/Akt, pMAPK/MAPK and GAPDH antibodies. Representative blots were shown for one of three independent experiments.

herceptin+EGF for 24 hours, the cell lysates were analyzed by Western blot to detect the degradation of ErbB2 and EGFR. The data were quantified by normalizing the ErbB2 and EGFR bands intensity to GAPDH using Odyssey system.

As shown in Figure 4.15 A, the peak representing surface ErbB2 shifted to the left to a small extent for all three treatments after a relatively long incubation time (24 hours), suggesting that herceptin induced a small amount of internalization of ErbB2. When comparing different treatments at the same time point, different doses of EGF did not enhance ErbB2 downregulation from the plasma membrane (Figure 4.15 B). The flow cytometry data of cells and cells treated with only secondary antibody were shown as negative controls. The corresponding immunoblots for ErbB2 and EGFR antibodies showed that the receptors were degraded when incubated with herceptin, EGF or herceptin+EGF (Figure 4.16 A). As shown in Figure 4.16 B and C, the normalized ErbB2 and EGFR levels suggested that herceptin or EGF alone induced some decrease in ErbB2 and EGFR but EGF alone induced a larger decrease. Herceptin and EGF together could induce the degradation of more than 70% of the receptors after 24 hours, which was much greater than when treated with herceptin or EGF alone. In conclusion, herceptin partially facilitated ErbB2 internalization and improved ErbB2 and EGFR degradation. Due to the time constraints, this experiment was carried out only once. These promising effects should be validated with additional replicate experiments. In addition, the impact of ankyrin105 expression in combination with herceptin+/-EGF should be tested. We might observe that EGFRs are further degraded in ankyrin105-expressing AU565 cells treated with herceptin.

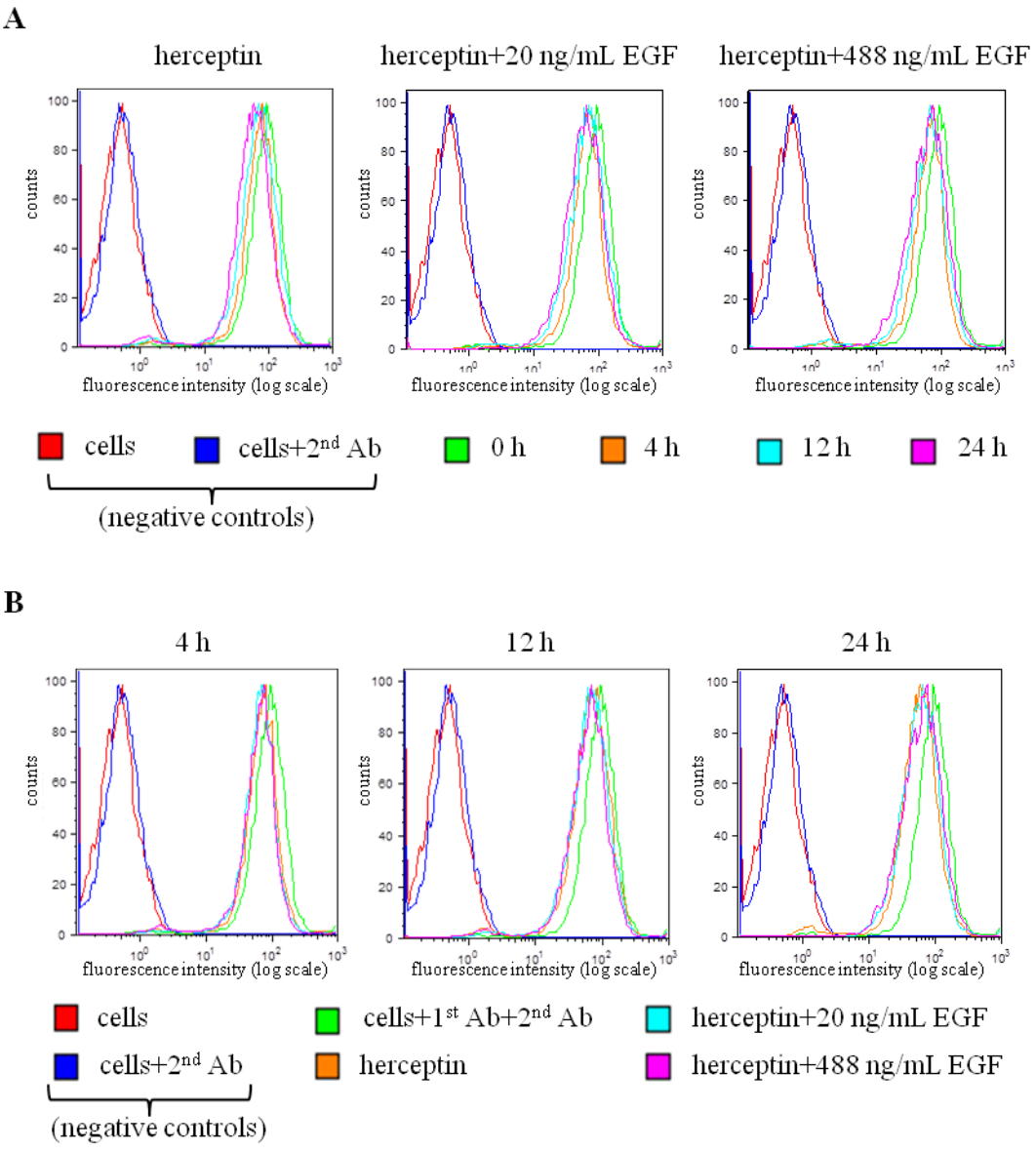

Figure 4.15 Effect of herceptin on surface ErbB2 in AU565 cells. AU565 cells were incubated with herceptin (10 μg/mL) and two different doses of EGF for various times at 37ºC. Flow cytometry analysis was used to detect the downregulation of ErbB2 from the plasma membrane. **A**, Data were analyzed by comparing different time points for the same treatment. **B**, Data were analyzed by comparing different treatments at the same time point. This experiment was carried out once.

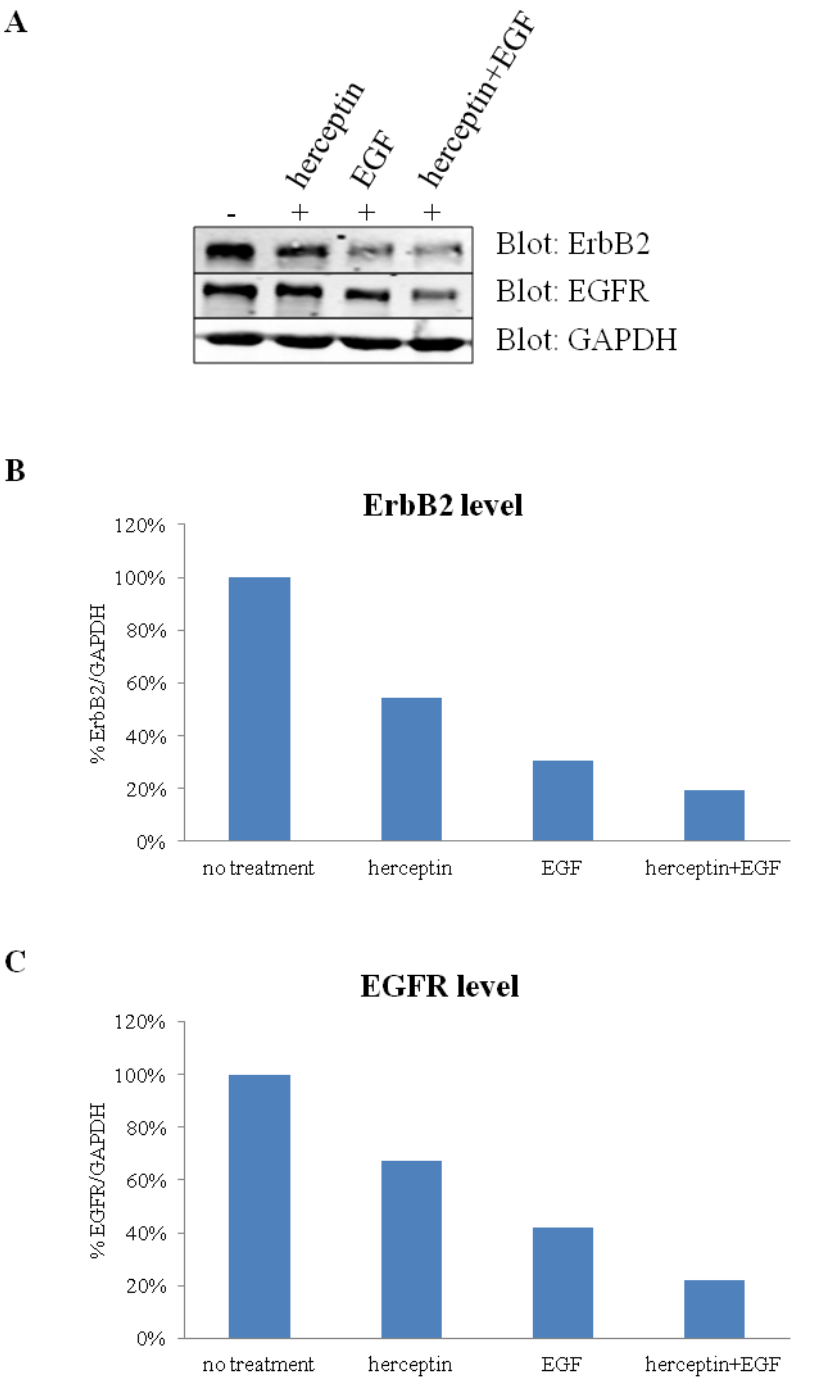

Figure 4.16 Effect of herceptin on ErbB2 and EGFR in AU565 cells. AU565 cells were incubated with herceptin (10 µg/mL), EGF and herceptin+EGF for 24 h at 37ºC. **A**, the cell lysates (25 µg of protein/lane) were analyzed by Western blot with EGFR, ErbB2 and GAPDH antibodies to detect the degradation of ErbB2 and EGFR. **B** and **C**, Western blot data were quantified by normalizing the ErbB2 and EGFR bands intensity to GAPDH using Odyssey system. These preliminary results were observed from one experiment.

5.0 DISCUSSION

5.1 Ankyrin3 Expression in Selected Cell Lines

Ankyrin3 is the most widely distributed among ankyrin family. Different isoforms are expressed in different cell types (Cunha and Mohler, 2006; Rubtsov and Lopina, 2000). We focused on its smaller isoform ankyrin105 because it has been demonstrated that overexpression of ankyrin105 promoted lysosomal-mediated degradation of the PDGFR in NIH 3T3 cells (Ignatiuk *et al*., 2006). The hypothesis of this project was that ankyrin105 would facilitate degradation of EGFR and enhance the downregulation of signaling pathways. Different cell types are thought to use different mechanisms for EGFR endocytosis and downregulation (Hendriks *et al*., 2003; Seto *et al*., 2002). Thus, we may observe different effects on EGFR levels upon ankyrin105 expression in different EGFR-expressing cell types. Therefore, five cell lines were chosen for this project. We first tested the endogenous expression of ankyrin105 in these selected cell lines. In COS-1, HEK293T and AU565 cells, there was relatively high expression of ankyrin120 and ankyrin105. Little or no ankyrin120 or ankyrin105 was detected in MCF10A cells and little ankyrin120 or ankyrin105 was present in MDA-MB-231 cells. In all 5 cell lines, the expression of large ankyrin3 isoform was relatively low (Figure 4.1). Thus, the cell lines used contained different levels of ankyrin3, but predominantly presented the 120 kDa and 105 kDa smaller isoforms when ankyrin3 was expressed. However, when we introduced ankyrin105 into each cell type during this study, we could not rule out the possibility that endogenous ankyrin105 in COS-1, HEK293T and AU565 cells might already facilitate the degradation of EGFR, such that adding more had little effect.

In the two breast cancer cell lines used in this project, MDA-MB-231 is a triple-negative (ER-, PR- and ErbB2-) breast cancer cell line (Tate *et al*., 2012), and AU565 is an ErbB2-positive (ER- or PR- and ErbB2+) breast cancer cell line (Lam *et al*., 2008). Based on ankyrin105 and ErbB2 expression in the two breast cancer cell lines (Figure 4.1), it could

suggest that ErbB2-positive breast cancer cell lines express high levels of ankyrin105 whereas triple-negative breast cancer cell lines express little or no ankyrin105. This needs to be tested further by examining ankyrin105 expression in a wide variety of breast cancer cell lines. Ankyrin105 expression in ErbB2-positive breast cancer cell line that also overexpresses EGFR (i.e. AU565) may reflect a cellular response to these overexpressed RTKs as the cells attempt to reduce receptor levels.

5.2 Overexpression of Ankyrin105 in Selected Cell Lines

5.2.1 Optimization of Transfection and Transduction Efficiency

In order to investigate the effect of overexpression of ankyrin105 on EGFR levels and downstream EGFR signaling, we needed transiently high expression of ankyrin105 during the time course of EGF stimulation. So we introduced ankyrin105 into 5 selected cell lines by either transfection of plasmid DNA or transduction using adenovirus. We optimized the conditions for calcium phosphate transfection and adenovirus transduction to get high expression of HA-Ank105. For calcium phosphate transfection, optimal amount of plasmid DNA was 30 μg for 10 cm plate (Figure 4.3). For adenovirus transduction, the optimal MOI of 200 (Table 4.1, Figure 4.7 and 4.8) was determined and EGF stimulation was carried out 4 or 5 days post-transduction (Figure 4.5).

5.2.2 Effect of Ankyrin105 Overexpression on Receptor Tyrosine Kinases and Signaling Pathways

Our laboratory has previously shown that overexpression of ankyrin105 promotes degradation of the basal PDGFRs and activated PDGFRs and causes a more sustained activation of the PI3K-Akt pathway, but does not affect the Ras-MAPK pathway in NIH 3T3 cells (Ignatiuk *et al*., 2006). Ankyrin105 may regulate multiple RTKs in the same way. We did not observe similar results in EGFR degradation and downstream signaling pathway downregulation in the 5 selected cell lines that transiently overexpressed ankyrin105.

In COS-1, HEK293T and AU565 cells, the degradation of basal EGFRs and activated EGFRs was not facilitated and downregulation of PI3K-Akt and Ras-MAPK pathways was not affected by ankyrin105 overexpression (Figure 4.2, 4.4 and 4.10). Thus, overexpression of ankyrin105 did not enhance EGFR degradation or downregulation of EGFR signaling pathways in these 3 cell lines. However, these cell lines all expressed relatively high levels of endogenous ankyrin105, which might already enhance EGFR downregulation to a certain extent. The introduced ankyrin105 may not be able to further improve EGFR degradation.

As compared to other selected breast cells, AU565 cells express high levels of ErbB2 (Neve *et al*., 2006). Unlike other EGFR family members, ErbB2 has a constitutively exposed dimerization arm, which can heterodimerize with other members of the EGFR subfamily, such as EGFR, even in the absence of growth factors (Burgess *et al*., 2003). ErbB2 is known to be internalization resistant, so EGFR-ErbB2 heterodimers may persist and prolong activation of receptors and downstream signaling pathways (Hommelgaard *et al*., 2004). ErbB2 can also enhance the binding affinity of the ligand (e.g. EGF) to its dimeric receptor (e.g. EGFR) (Fuller *et al*., 2008). Therefore, the overexpression of ErbB2 in AU565 cells may block internalization, endocytosis and degradation of receptors and prolong signaling pathways. As shown in Figure 4.10, EGFRs were not degraded upon EGF stimulation. This was probably because ErbB2 heterodimerized with EGFRs and inhibited EGFR downregulation. Ankyrin105 could not impact EGFR degradation since EGFR-ErbB2 heterodimers were not internalized.

We also observed that Akt activation was upregulated in both HA and HA-Ank105 transfected HEK293T cells while Akt and MAPK activation were downregulated in both Ad-GFP and Ad-GFP+HA-Ank105 transduced AU565 cells, compared to their parental cells, respectively (Figure 4.4 and 4.10). For calcium phosphate transfection, the mechanism regulating the uptake of the precipitate containing calcium phosphate and DNA is still unknown (Batard *et al*., 2001; Kingston *et al*., 2001). For adenovirus transduction, the gene delivery is via episomal expression (Hendrie and Russell, 2005). Adenovirus uses both clathrin-mediated and caveolae-mediated endocytosis to enter the cells (Campos and Barry, 2007; Smith and

Helenius, 2004). The uptake of adenovirus results in induction of intracellular signaling, such as the activation of the Ras-MAPK pathway and the PI3K-Akt pathway (Greber, 2002; Liu and Muruve, 2003). Therefore, calcium phosphate transfection and/or adenovirus transduction processes might affect both endocytosis and intracellular signaling pathways in ways not well understood.

In MCF10A and MDA-MB-231 cells, the degradation of basal EGFRs and activated EGFRs was not facilitated and downregulation of PI3K-Akt and Ras-MAPK pathways was not affected by ankyrin105 overexpression (Figure 4.6 and 4.8). Thus overexpression of ankyrin105 did not enhance EGFR degradation or downregulation of EGFR signaling pathways in these 2 cell lines as well as the other 3 tested cell lines. In MDA-MB-231 cells, EGFRs were not degraded in response to EGF stimulation, which suggested EGFR degradation pathway might be defective and this cell line was not suitable to study ankyrin105 effects on EGFR degradation. The downregulation of activated EGFRs and activated MAPK was facilitated in both Ad-GFP and Ad-GFP+HA-Ank105 transduced cells compared to parental cells. This also suggested that adenovirus transduction process might affect intracellular signaling pathways.

There might be several reasons why ankyrin105 did not impact EGFR signaling. First, ankyrin105 may not regulate degradation of EGFR or enhance the downregulation of signaling pathways at all. Second, the selected cell lines had some limitations, such as endogenous ankyrin105 expression and ErbB2 expression, or may contain an intrinsic EGFR downregulation defect, which could block the effect of overexpressed ankyrin105. Third, the number of cell lines chosen for this study may not have been large enough to find cell lines that possessed the criteria necessary for ankyrin105 to play a role in EGFR signaling. Fourth, perhaps the transfection and/or transduction procedures temporarily interfered with ankyrin105 effects by perturbing internalization, endocytosis and trafficking pathways such that stable cell lines expressing ankyrin105 will need to be tested well after transfection or transduction. The effect of ankyrin105 overexpression on RTKs and signaling pathways still remains unclear since only PDGFR degradation in NIH 3T3 cells and EGFR degradation in 5 selected cells

were tested. The effect of ankyrin105 might be cell type specific (NIH 3T3 cells, but not COS-1, HEK293T, MCF10A, MDA-MB-231 or AU565 cells) or RTK specific (PDGFR, but not EGFR). Further experiments need to be carried out to evaluate ankyrin105 overexpression in a variety of cell lines under stable cell line conditions.

5.3 Effect of Cancer Therapeutics in AU565 Cells

The high expression of ErbB2 in AU565 cells promotes EGFR-ErbB2 heterodimer formation (Fuller *et al*., 2008). Due to resistant internalization of ErbB2, the endocytosis of ErbB2-containing heterodimers is inhibited, which may result in upregulation of signaling pathways (Hommelgaard *et al*., 2004; Yarden and Sliwkowski, 2001). Overexpression of ErbB2 in AU565 cells blocks degradation of EGFR, which makes it difficult to test ankyrin105 effect on EGFR and its downstream signaling. Therefore, ErbB2 needed to be downregulated before the study of ankyrin105 could be carried out. In this project, 2 drugs were used to facilitate ErbB2 downregulation.

5.3.1 Effect of Geldanamycin Treatment on Receptor Tyrosine Kinases and Signaling Pathways

Geldanamycin is an Hsp90 inhibitor. It can bind to and inactivate the conserved ATP/ADP-binding pocket of Hsp90, and destabilize Hsp90 client proteins (e.g. ErbB2, IR, p53, Akt and MEK) (Austin *et al*., 2004; Pedersen *et al*., 2009; Zuehlke and Johnson, 2010). To investigate internalization of ErbB2 by geldanamycin, flow cytometry analysis was used to measure cell surface ErbB2 (Figure 4.11). Geldanamycin induced a decrease in surface ErbB2 and the internalization was increased as the incubation time was prolonged. EGF did not facilitate ErbB2 downregulation of surface ErbB2 or further improve geldanamycin-induced ErbB2 internalization. To investigate the degradation of ErbB2 by geldanamycin, Western blot analysis was used to detect the total levels of ErbB2 (Figure 4.12). It showed that both ErbB2 and EGFRs were degraded in response to geldanamycin treatment. The EGFR degradation

resulted from the decreased ErbB2 levels after incubation with geldanamycin. Similarly, EGF treatment did not change the receptors level and GA+EGF treatment did not further promote the degradation of receptors. Therefore, as shown in the literature, geldanamycin could induce degradation of ErbB2.

Then the ability of ankyrin105 overexpression to enhance receptors degradation in combination with geldanamycin treatment was assessed. Flow cytometry analysis was no longer suitable for this study. The fluorescent light from GFP (co-expressed by the adenovirus) in transduced cells had a certain degree of overlap with that from the PE signal, which made the PE peak shift to the left and show false positive results. As shown in Western blot results (Figure 4.13 and 4.14), ankyrin105 did not impact EGFR degradation or its downstream signaling pathways in geldanamycin-treated AU565 cells which already downregulated ErbB2 and EGFR. These results suggested that overexpression of ankyrin105 did not further enhance EGFR degradation or downregulation of EGFR signaling pathways as the levels of EGFR were rapidly reduced at the same time as geldanamycin induced ErbB2 degradation.

As an Hsp90 inhibitor, geldanamycin may promote degradation of Hsp90 client proteins including p53 and Akt that are important for multiple cellular processes. Thus, incubation of cells with geldanamycin has toxic side effects and may interrupt normal cellular mechanisms. Although geldanamycin can induce ErbB2 degradation, it is still not used for clinical cancer therapeutics mainly because of these toxicities. Perhaps testing lower concentrations of geldanamycin would reduce these toxicities and the addition of ankyrin105 expression could make it more selective for ErbB2 and EGFR degradation.

5.3.2 Effect of Herceptin Treatment on Receptor Tyrosine Kinases

Herceptin is a monoclonal antibody that binds the ErbB2 extracellular domain, blocks ErbB2-induced dimerization and inhibits intracellular tyrosine kinase activity (Hudis, 2007; Tsang and Finn, 2012). It can enhance ErbB2 internalization and degradation, and regulate intracellular downstream signaling pathways (Spector and Blackwell, 2009). As shown in

Figure 4.15, herceptin induced a small amount of internalization of ErbB2. Different doses of EGF did not have an effect on ErbB2 internalization in combination with herceptin. The Western blot results showed that both ErbB2 and EGFRs were degraded when incubated with herceptin, EGF or herceptin+EGF (Figure 4.16). Cells treated with both herceptin and EGF could induce much more degradation of the receptors compared to those treated with herceptin or EGF alone. These results suggested that herceptin improved ErbB2 and EGFR internalization and degradation. It was strange that the amount of ErbB2 degraded was more than internalized (Figure 4.15 and 4.16). These experiments only carried out once and replicate experiments are needed to confirm the conclusions.

In clinical treatment, patients may become herceptin resistant (Gajria and Chandarlapaty, 2011) and have some side effects such as cardiac toxicity, neutropenia and anemia (Chen *et al*., 2011; Chien and Rugo, 2010). Besides, the full course of herceptin treatment is expensive. So new therapeutics may be developed by using less herceptin in combination with EGF based on the preliminary results of Figure 4.16.

5.4 Future Directions

In this project, we performed growth factor stimulation to evaluate ankyrin105 effects on EGFR levels and intracellular signaling in 5 selected cell lines. Our goal was to reduce EGFR levels and signaling in breast cancer cells and evaluate the effect on cancer cell phenotypes. Since EGFR levels were largely unaffected by ankyrin105 expression, the cancer cell properties were not analyzed. Cancer cells usually display a decrease in both cell-cell and cell-matrix attachment and apoptosis and an increase in metastasis and proliferation (Kim *et al*., 2011; Weiss and Ward, 1983). Thus, several other experiments can be carried out to test the cancer cell properties in ankyrin105-overexpressing cells, such as adhesion, apoptosis, migration and proliferation assays. These additional results may provide a better understanding of how ankyrin105 impacts signaling regulation and the tumorigenic properties in these cell lines.

In this project, we did not observe an effect of ankyrin105 overexpression on EGFR

degradation and its downstream signaling. Based on the results of Ignatiuk *et al*. and this project, the effect of ankyrin105 might be cell type specific or RTK specific. Further experiments to study ankyrin105 effect on EGFR degradation need to be carried out in a larger variety of cell lines. A suitable cell line for this study should express little or no endogenous ankyrin105 and ErbB2 and express high endogenous EGFR that can be degraded in response to EGF stimulation. When introducing ankyrin105 into cells, we need to consider that the gene delivery process may impact internalization, endocytosis and intracellular signaling pathways. So a method that minimizes this impact and expresses high levels of the ankyrin105 protein needs to be selected. For example, stable cell lines expressing ankyrin105 can be generated well after transfection or transduction. If differential effects on degradation kinetics of EGFR are observed, we could characterize the different mechanisms during EGFR endocytosis (e.g. clathrin-dependent or caveolae-dependent) to understand how ankyrin105 takes part in the receptor degradation process. We can also introduce EGFR into NIH 3T3 cells since NIH 3T3 doesn't express EGFR, and then test whether ankyrin105 overexpression has the same effect on EGFR degradation as previously shown for PDGFR degradation.

For AU565 cells, herceptin treatment experiments need to be repeated to confirm the preliminary results. We can also try different concentrations of herceptin with the combination of EGF to optimize experiment conditions. A time course of EGF stimulation in parental cells, cells transduced with Ad-GFP and Ad-GFP+HA-Ank105 can be carried out after herceptin treatment to assess whether ankyrin105 overexpression can further downregulate EGFR. Alternatively, it might be best to generate stable AU565+HA-Ank105 cell lines and then compare their behavior upon herceptin+/-EGF with the parental AU565 cells.

5.5 Conclusion

The overexpression of ankyrin105 did not enhance EGFR degradation or downregulation of EGFR downstream signaling (PI3K-Akt and Ras-MAPK pathways) in COS-1, HEK293T, MCF10A, MDA-MB-231 and AU565 cells. The transfection or transduction process might

affect internalization, endocytosis and intracellular signaling pathways.

In AU565 cells, geldanamycin induced the internalization and degradation of ErbB2 while EGF did not facilitate ErbB2 downregulation or further improve geldanamycin-induced ErbB2 downregulation. EGFRs were also degraded in response to geldanamycin treatment. Ankyrin105 overexpression did not impact EGFR degradation or its downstream signaling pathways in geldanamycin-treated cells. Herceptin induced a small amount of internalization and degradation of ErbB2. EGF did not have an effect on ErbB2 internalization in combination with herceptin, but further promoted herceptin-induced ErbB2 downregulation. EGFRs were degraded when incubated with herceptin, EGF or herceptin+EGF, but degraded to a higher extent with herceptin+EGF treatment.

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